Drugs and drug-like molecules can modulate mucosal-associated invariant T cell function

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Running title: MAIT cells, drugs and drug-like molecules

Abstract

MR1 can present MAIT cell activating and non-activating vitamin B-based ligands. Whether MR1 binds other ligands is unknown. Here we identify a range of small organic molecules, drugs, drug metabolites and drug-like molecules, including salicylates and diclofenac, as MR1-binding ligands. Some of these ligands inhibited MAIT cells *ex vivo* and *in vivo*, while others, including diclofenac metabolites, were agonists. Crystal structures of a MAIT TCR complexed with MR1 bound to the non-stimulatory and stimulatory compounds showed distinct ligand orientations and contacts within MR1, highlighting versatility of the MR1 binding pocket. The findings indicate that MR1 can capture chemically diverse structures, spanning mono- and bicyclic compounds, which either inhibit or activate MAIT cells. This demonstrates that drugs and drug-like molecules can have the previously unknown property of modulating MAIT cell function in mammals.

Introduction

In humans, the *human leukocyte antigen* (*HLA*) *locus* is highly polymorphic, thereby enabling HLA molecules to present a broad array of peptide antigens (Ags), an essential requirement for protective immunity. Conversely, certain HLA allotypes are often associated with aberrant T cell reactivity, including autoimmunity, food and drug-linked hypersensitivities[1, 2]. For example, the anti-retroviral drug abacavir binds to HLA-B*57:01 and alters the repertoire of bound peptides, leading to abacavir hypersensitivity syndrome[3]. Humans also possess an array of monomorphic Ag-presenting molecules that play distinct and specialised roles in immunity[4]. Namely, the CD1 family is well suited to bind lipid-based Ags[5], while the MHC class I-related molecule, MR1, presents microbial products associated with vitamin B2 synthesis for recognition by mucosal-associated invariant T (MAIT) cells [6]. Whether MR1 or CD1 can present small molecule drugs or drug-like molecules to induce an immune response remains unclear, with such potential implications being magnified by the monomorphic nature of these Ag-presenting molecules.

MAIT cells are an evolutionary conserved innate-like T cell subset that is found in many mammals[7, 8]. Although the function of MAIT cells is emerging, they are considered to play key roles in immunity[9 10, 11]. MAIT cells are abundant in humans, representing up to 10% of the total T cell population in the blood. They are found concentrated at mucosal sites, and can account for up to 45% of the total T cell population in the liver, a major detoxifying organ that is directly linked to the gastrointestinal system [12]. Unlike MHC-restricted T cell receptors (TCRs) that typically display a diverse repertoire, MAIT cells mostly

express a TCR that possesses an 'invariant' TCR α-chain (TRAV1-2-TRAJ33/20/12) paired with a limited array of TCR β-chains[8, 13, 14]. MR1 can present stimulatory and non-stimulatory pyrimidines associated with vitamins B2 and B9, respectively, for MAIT cell surveillance. For example, some uracils and lumazines derived from riboflavin biosynthesis activate MAIT cells, whereas derivatives of folic acid, including 6-formylpterin (6-FP), are non-stimulatory ligands[15, 16]. A key difference between these stimulatory and non-stimulatory antigens is that the former possess a ribityl chain that mediates contacts with the MAIT TCR[17]. Upon activation via the MAIT TCR, MAIT cells rapidly secrete an array of pro-inflammatory cytokines, proliferate and have the capacity to kill bacterially infected cells[18, 19]. It is therefore important to understand the range of ligands that can modulate MAIT cell function.

Using parallel *in silico* discovery approaches, we identified potential MR1-binding ligands, including some commonly prescribed drugs and drug-like molecules, and other simple aromatic aldehydes, ketones, carboxylates and analogues, pyrimidines, quinones and flavones. Further, we evaluated these ligands for their ability to modulate MAIT cell function through their interaction with MR1. We found that some caused MR1 cell surface upregulation and inhibited MAIT cell activation, while others activated MAIT cells. Consequently, we demonstrate that MR1 can capture chemically diverse scaffolds. These observations indicate that some drugs and drug-like molecules impact MAIT cell function.

Results

In silico prospecting for MR1 ligands

Our previous studies identified uracil analogues including 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and pterin/pteridine analogues like 6-formylpterin (6-FP) as agonists and inhibitors, respectively, of MAIT cell activation[15]. These compounds contain simple pyrimidine heterocycles as scaffolds, suggesting that the ligand-binding site of MR1 (the A´-pocket) has a propensity to bind small molecules, possibly including drugs, drug-like molecules or fragments. Here, the A´-pocket of MR1 features an unusual Schiff base-forming Lys43, sitting at the base of an aromatic cradle within MR1 that is ideal for accommodating aromatic ligands, and a network of polar sidechains (e.g. Arg9, Ser24, Arg94) that can interact with both a uracil and a ribityl chain[16].

To identify other chemical scaffolds capable of binding to MR1, we conducted multiple *in silico* screens in parallel using different chemical libraries, including 6,000 in-house organic compounds and 1,216 FDA approved drugs from the Distributed Structure-Searchable Toxicity (DSSTox) database to target the A´-pocket (**Figure 1**). Based on MR1-binding by the most active known MAIT cell antigen (5-OP-

RU), we performed fragment-based virtual screening of the 6,000 compound library to identify small aromatic aldehydes or carboxylates that might neutralize Lys43, as well as simple pyrimidines, quinones and enones (**Fig. 1a**), to bind at the same site as 5-OP-RU (**Fig. 1b**). This led to 147 hits, including those shown (**Fig. 1c**). Additionally, a database of 1,216 FDA approved drugs was screened *in silico* (**Fig. 1d**) for shapes matching to the ligands 6-FP (**Fig. 1e**) and 5-OP-RU (**Supplementary Figure 1a**), leading to 16 virtual hits including those shown (**Fig. 1f**). A third *in silico* approach involved receptor-based virtual docking (**Fig. 1g**) of a subset of 470 of 1,216 FDA approved drugs (MW 150-300 Da) into the ternary crystal structure of 5-OP-RU bound MR1-MAIT TCR (**Fig. 1h and Supplementary Figure 1b & c**). This led to identification of 20 virtual hits, including those shown (**Fig. 1i**). In summary, these parallel approaches (**Figure 1)** produced 183 distinct hits with chemically diverse structures (**Supplementary Tables 1 and 2**), suggesting that MR1 has the potential to bind to many structurally different compounds.

Diverse MR1 ligands can inhibit or activate MAIT cells

Using *in vitro* functional assays, we evaluated 81 of these *in silico* hits for their ability to bind to MR1 and to stimulate MAIT cells. We hypothesized that some might be like 5-OP-RU, binding to MR1 and activating MAIT cells; others might be like Ac-6-FP and 6-FP, binding MR1 but not activating MAIT and acting as competitive inhibitors of MAIT cell stimulation by 5-OP-RU. MR1 binding and stimulatory capacity of the compounds were assessed in experiments whereby an antigen presenting cell line overexpressing MR1 (C1R.MR1) and a T cell line overexpressing a MAIT TCR (Jurkat.MAIT-A-F7, TRBV6-1 TCR) were co-incubated with the test compound. MR1 binding was measured by increased MR1 cell surface expression on C1R.MR1 cells. Stimulatory capacity was measured by increased CD69 surface expression on Jurkat.MAIT-A-F7 cells. As summarized (**Supplementary Table 3**, **Figure 2a and Supplementary Figure 2a**), approximately a quarter of the compounds had a measurable effect on MR1 upregulation and/or MAIT cell stimulation, including 9 drugs, 5 flavones and 8 small molecule compounds (**Supplementary Figure 3**). Of the 16 compounds that upregulated MR1, 8 were agonists (**Supplementary Figure 2a**) (**Figure 2a**). Thus, whilst MR1 can bind to diverse chemical ligands (**Supplementary Figure 3**), the requirements relating to MAIT cell activation were more stringent. Notably, many of the aromatic aldehydes/ketones/carboxylic acids (**Figure 1a and Supplementary Tables 1–3**) that fitted into MR1 did not upregulate MR1 cell surface expression appreciably, indicating that simply neutralizing Lys43 through either Schiff-base formation or an ionic interaction was insufficient for MR1 binding and that other binding components were also required.

We then compared 6-FP and Ac-6-FP with a selection of the compounds that strongly upregulated MR1, but were not agonists, for their MR1 binding and inhibitory capacity. These included the drug related compounds 3-formylsalicylic acid (3-F-SA), 5-formylsalicylic acid (5-F-SA) and 2-hydroxy-1-naphtaldehyde (2-OH-1-NA) (**Figure 2b**). Kinetics and levels of MR1 upregulation of these three compounds were similar as compared to 6-FP (**Figure 2c, Supplementary Figure 2b**). 3-F-SA and 2-OH-1-NA also effectively inhibited MAIT cell activation in the presence of 5-OP-RU in a dose-dependent manner, as judged by CD69 upregulation assays with three Jurkat.MAIT cell lines (Jurkat.MAIT-A-F7, Jurkat.MAIT-#6 and Jurkat.MAIT-C-F7) (**Figure 2d and Supplementary Figure 2c**). As judged by half maximum inhibitory concentrations (IC_{50}) across the range of 5-OP-RU doses tested (**Supplementary Table 4**), we established that Ac-6-FP was the most potent inhibitor, followed by 3-F-SA and 6-FP which were comparable in their ability to inhibit MAIT activation, with 2-OH-1-NA being the least potent inhibitor. A similar pattern was observed for IL-2 production by a mouse MAIT TCR transduced BW58 cell line (BW58.CD3.MAIT-Vβ8.2) (**Figure 2e and Supplementary Figure 2d, Supplementary Table 4 for IC_{50} values**). Notably CD69 upregulation by the T cell lines in response to phorbol 12-myristate 13-acetate (PMA)/Ionomycin stimulation was not impaired in the presence of these inhibitors (**Supplementary Figure 2e**); similarly simulation of a conventional T cell line by its cognate Ag was not impaired (**Supplementary Figure 2e**) thus indicating that the inhibitory effects were specific to the MAIT-MR1 axis.

Interestingly, the fine specificity underpinning Jurkat.MAIT cell activation and inhibition towards chemically similar structures was evident when comparing 3-F-SA and 5-F-SA. Namely, while 3-F-SA did not stimulate any of the three Jurkat.MAIT-TCR cell lines, 5-F-SA showed modest activation of Jurkat.MAIT-#6 (TRBV6-4) that was blocked by anti-MR1 26.5, but 5-F-SA did not activate Jurkat.MAIT-A-F7 (TRBV6-1) or Jurkat.MAIT-C-F7 (TRBV20, not shown) (**Figure 2f**), indicating that MAIT TCR β-chain usage can fine tune the responsiveness to certain ligands.

A drug degradation product binds and upregulates MR1

The antifolates, aminopterin and its closely related derivative chemotherapeutic methotrexate, are used to treat certain cancers and autoimmune disorders, including rheumatoid arthritis and psoriasis[20]. Aminopterin readily photodegrades in UV light to form 2,4-diamino-6-formylpteridine (2,4-DA-6-FP). While methotrexate is more stable, it nevertheless forms the same photodegradation product [21]. Given the similarity between the naturally occurring photodegradation products, 6-FP and 2,4-DA-6-FP

(**Supplementary Figure 4a**), we reasoned that the latter molecule might also upregulate MR1 cell surface expression. Indeed, photodegradation preparations of aminopterin sponsored MR1 refolding and upregulated MR1 cell surface expression, while untreated aminopterin did not (**Supplementary Figure 4b-d**). Mass spectrometry analysis comparing the captured antigen with synthetically produced compound, showed that 2,4-DA-6-FP was captured within the refolded MR1 molecule (**Supplementary Figure 4e**). Collectively, these studies indicated that the metabolite 2,4-DA-6-FP is a novel MR1 ligand, subsequently confirmed by structural studies (see below).

Diclofenac metabolites can activate MAIT cells

We also characterized an agonistic drug identified from the functional screen, diclofenac (DCF), a commonly prescribed non-steroidal anti-inflammatory drug. Whilst not apparently upregulating MR1, DCF activated Jurkat.MAIT-A-F7 cells, with this activation being blocked by the anti-MR1 monoclonal antibody 26.5 and competitively inhibited by Ac-6-FP in a dose dependent manner (**Figure 3a**). The level of stimulation reached at 100 μM ligand concentration was approximately 2-fold greater than that of the ribityllumazine agonists, 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-DiMe) and 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH)[16] and comparable to maximum stimulation levels reached with 5-OP-RU, albeit at a 1 x 10^6 fold lower concentration (**Figure 3a**). Interestingly, while DCF activated Jurkat.MAIT cells expressing the A-F7 MAIT TCR (using TRBV6-1), it did not activate two other Jurkat.MAIT cell lines (#6, and C-F7), expressing TRBV6-4 or TRBV20 TCR β-chain segments, indicating that the agonistic properties of DCF are dependent on the TCR β-chain (**Figure 3b**) suggestive of MAIT cell subset specific activation. Moreover, 5-OP-RU activation of Jurkat.MAIT-A-F7 cells was increased in the presence of DCF, and DCF did not inhibit 5-OP-RU activation of Jurkat.MAIT-#6 and Jurkat.MAIT-C-F7 cells (**Figure 3c**).

To understand if DCF was broadly stimulatory to MAIT cells we tested a panel of 12 cell lines expressing MR1 restricted TCRs [13, 22] that varied in TCR α- and β-chain usage as well as reactivity to 5-OP-RU, 6-FP, Ac-6-FP and absence of ligand to DCF in the presence of C1R.MR1 cells (**Figure 3d and Supplementary Figure 5**). Given that DCF is known to be metabolised, we tested if the observed stimulation was due to DCF itself or three of its hydroxyl metabolites (4-hydroxy, 5-hydroxy, 4,5-dihydroxy diclofenac) and two glucuronide conjugates (diclofenac acyl-β-D-glucuronide and diclofenac acyl-β-D-glucuronide allyl ester) (**Figure 3d-e**). Importantly DCF and its metabolites did not modulate stimulation of a conventional T cell line by its cognate Ag (**Supplementary Figure 5b**) assuring

specificity of the observed responses. Of the cell lines tested, the A-F7 TCR was specifically stimulated by DCF. Interestingly, for this cell line stimulation by 5-OH-DCF was stronger than DCF itself. Similarly, 10 other of the 12 cell lines tested responded modestly to 4′-OH-DCF whilst none of the other DCF metabolites yielded specifically increased responses. DCF responsive cell lines included two (MAV36 and MAV21) that expressed atypical TRAV1-2- MR1-restricted TCRs, suggesting that recognition of these metabolites extends beyond classical MAIT cells. Collectively, these results indicated that observed MAIT cell activation by DCF might be more attributable to one of its metabolites.

MR1 cell surface presentation of drugs and drug-like molecules

Given that many drugs are metabolized within cells, we next asked whether some of the compounds that inhibited or activated MAIT cells had been metabolized in antigen-presenting cells (APCs) before being presented by MR1 at the cell surface. To establish this, we immunoprecipitated MR1 from the surface of C1R.MR1 cells and conducted mass spectrometry analyses of the ligands captured by MR1. To validate this approach, we incubated the C1R.MR1 cells with Ac-6-FP, and specifically detected this ligand in the MR1 eluate fraction (**Figure 3f**). Using this approach, we also determined that 3-F-SA was presented by MR1 on the cell surface (**Figure 3f**). Next, we established that it was DCF metabolites, and not DCF itself, that were presented on the cell surface. Namely, metabolites of DCF bound to MR1 on the cell surface included hydroxylated DCF (either 5-OH-DCF, 4'-OH-DCF, which are indistinguishable via mass spectrometry, or a combination of the two) and 4',5-diOH-DCF, which was subsequently confirmed by mass spectrometric analyses of the pure, chemically synthesized DCF metabolites (**Figure 3f**). DCF and the glucuronated species were not detected, which could be due to cell type specific metabolism or the larger size of glucuronated DCF that no longer fits the ligand-binding pocket of MR1. Accordingly, drugs, drug-like molecules as well as their metabolites can be presented by MR1 to MAIT cells.

***Ex vivo* modulation of MAIT cell activity**

Next, we established the extent to which three of these newly identified MR1 ligands (3-F-SA, 5-F-SA, 2-OH-1-NA, and controls, 6-FP and Ac-6-FP) could modulate MAIT cell activity *ex vivo*. MAIT cells, identified as MR1-5-OP-RU tetramer$^+$ TRAV1-2$^+$ lymphocytes within the CD3$^+$ population of PBMCs from five donors, were examined for the competitive inhibition of the drugs and drug-like molecules on 5-OP-RU dependent MAIT cell activation, assaying for TNF and IFNγ cytokine production as activation markers (**Figure 4a**). The compounds inhibited MAIT cell activation in a dose-dependent manner, with Ac-6-FP representing the most potent competitive inhibitor, followed by 3-F-SA. Less potent inhibitor

effects were observed for 2-OH-1-NA, 5-F-SA and 6-FP. We also examined the ability of these compounds to inhibit MAIT cell proliferation when incubated with 5-OP-RU. The pattern of inhibitory effects on cytokine production mirrored the inhibitory effects on cell proliferation (**Figure 4b**). Importantly, MAIT cells and TRAV1-2^{+} non-MAIT cells produced cytokine in response to PMA/Ionomycin stimulus (**Supplementary Figure 6a**) and proliferated in response to plate bound CD3-CD28 stimulus (**Supplementary Figure 6b**) regardless of the presence of inhibitory molecules, indicating that toxicity is unlikely to simulate inhibition. Accordingly, drugs and drug-like molecules can impact on MAIT cell activity *ex vivo*.

***In vivo* modulation of MAIT cell activity**

Further, we next established the efficacy of two of these ligands to inhibit MAIT cell proliferation *in vivo* using a previously established MAIT cell model in C57BL/6 mice [23]. In this model synthetic 5-OP-RU in conjunction with TLR stimulus causes MAIT cells to accumulate in the lungs to make up to about 25-50 % of αβT cells as compared to 0.5-2 % in the naïve mouse. TLR stimulus can either be provided by synthetic TLR agonists such as Pam2Cys (TLR2/6) [24], poly I:C (TLR3), or CpG (TR9) or by bacteria deficient in the riboflavin pathway, as we showed for the live-attenuated vaccine strain of *Salmonella enterica* serovar Typhimurium, *S.* Typhimurium BRD509, deficient in riboflavin synthesis (Salm.BRD509Δ*ribDH*).

Mimicking a model of infection whereby 5-OP-RU is extracellularly supplied for Ag presentation in conjunction with TLR stimulus from the intracellular Salm.BRD509Δ*ribDH* bacteria, repeated application of Ac-6-FP or 3-F-SA inhibited MAIT cell accumulation in the lungs in a dose dependent manner (**Figure 4c**). Importantly Ac-6-FP or 3-F-SA had no impact on bacterial CFU counts, absolute numbers of non-MAIT αβ T cells and IL-17 production by non-MAIT αβ T cells in the lungs in response to Salm.BRD509Δ*ribDH* (**Supplementary Figure 7**), indicating that the inhibitory effects were not due to toxic effects by the inhibitors on bacteria or T cells. Accordingly, these inhibitors can effectively abrogate MAIT cell activation *in vivo*.

Structures of MAIT TCR-MR1-ligand complexes

We sought to determine how these drugs, their derivatives, and drug-related compounds could be accommodated within the MR1 binding pocket and modulate MAIT cell activity. Accordingly, we determined the crystal structures for the A-F7 (TRBV6-1) MAIT TCR bound to MR1 presenting non-

stimulatory ligands (2,4-DA-6-FP; 2-hydroxy-5-methoxybenzaldehyde (HMB); 2-OH-1-NA; and 3-F-SA) and two activating ligands (DCF and 5-OH-DCF) to high resolution (**Supplementary Table 5**). All drugs and drug-related compounds were clearly visible within the MR1-Ag-binding cleft (**Supplementary Figure 8**), thereby permitting detailed atomic insight into the mechanism underpinning their effect on the MAIT-MR1 axis. The MAIT TCR docked atop the six MR1-drug/drug-like complexes with a very similar topology (**Supplementary Figures 8g & 9)**. Here, the root mean square deviation, the buried surface area (BSA) values and relative contributions of the CDR loops between the complexes were very similar (**Supplementary Table 6 & Supplementary Figure 9**). Thus recognition of diverse chemical scaffolds does not translate to diverse MAIT TCR-MR1 docking, thereby underscoring the innate-like recognition properties of the MAIT TCR. Further, all of the drugs and drug-like molecules were located within the A´-pocket of MR1 (**Figures 5 and 6**), which not only validates the *in silico* approaches (**Figure 1 & Supplementary Figure 10**), but formally demonstrates that this pocket is sufficiently versatile to accommodate distinct chemical identities. All of these non-stimulatory ligands formed a Schiff base with Lys43 at the base of the aromatic cradle **(Figures 5a-e**), and, consistent with their non-stimulatory properties, none of them directly contacted the MAIT TCR. 2,4-DA-6-FP was located in essentially an identical position as 6-FP (**Figures 5a & b**), where the pteridine ring of 2,4-DA-6-FP was sandwiched between Tyr7 and Trp69, and formed additional aromatic pi-interactions with Trp156 and Tyr62, whilst abutting Ile96 (**Figure 5b**). The difference between 6-FP and 2,4-DA-6-FP is the presence of a carbonyl group in the former versus an amino group in the latter at position-4 of the pteridine ring. This difference impacts at the base of the cleft, with either the carbonyl or amino group nestling against Arg9 in the MR1-6-FP complex (**Figures 5a & b**). The latter interaction is unfavourable and likely accounts for weaker MR1 upregulation (**Supplementary Figure 4b & c**). Accordingly, subtle chemical differences between ligands can impact on the associated MR1-binding properties.

The compound 2-OH-1-NA, like 6-FP, possesses a bicyclic ring structure, and while the planes of the aromatic rings are similarly disposed, 2-OH-1-NA is displaced by ≈ 4Å towards the α1-helix of MR1 (**Figure 5c**). In adopting this binding mode, its 2-hydroxy group, the only polar moiety of 2-OH-1-NA, hydrogen bonds to His58, and packs against Tyr162, while the interactions with Arg9, Arg94, and Trp69 are lost (**Figure 5c**). 3-F-SA and HMB are monocyclic (**Figures 5d & e**), with the latter compound being directly superposable over 2-OH-1-NA, thereby mediating similar interactions with MR1, including the hydrogen bond with His58 (**Figures 5d, e & f**). The 5-methoxy group is not involved in polar interactions, but is stabilized by packing against Tyr7 (**Figures 5d**). In contrast, 3-F-SA is rotated in the same plane by approximately 70° around the Schiff base in comparison to 2-OH-1-NA, and accordingly

is accommodated in a different region of the A´-pocket of MR1 (**Figure 5e**). Namely, the carboxyl group of 3-F-SA leaned over to salt bridge with Arg9 and hydrogen bond to Ser24, while its aromatic ring is wedged between Tyr7 and Trp69 (**Figure 5e**). These additional interactions are consistent with 3-F-SA representing a potent inhibitor of MAIT cell function **(Figure 2c)**. Accordingly, there is sufficient plasticity within the A´-pocket of MR1 to accommodate diverse chemical scaffolds.

MAIT TCR-MR1-stimulatory ligand complexes

DCF stimulated Jurkat.MAIT cells in an apparent subset specific manner (**Figure 3**), but its stimulatory activity appeared attributable to its metabolites, including 5-OH-DCF and 4'-OH-DCF (**Figure 3f**). Comparing the crystal structures of the MAIT TCR bound to MR1 presenting DCF and 5-OH-DCF provided insight into the agonist properties of 5-OH-DCF (**Figures 6a-f, Supplementary Tables 7 & 8**). Neither species formed a Schiff base with MR1 (**Figures 6d & e**), consistent with the poor ability of DCF (and metabolites thereof) to upregulate MR1, and reminiscent of the weak agonist RL-6-Me-7-OH that also did not form a Schiff base with MR1[17]. Despite this, the positioning of Lys43 remained relatively well fixed within the MR1 cleft, forming a hydrogen bond with the adjacent His58 (**Figures 6d & e**). In contrast to the non-stimulatory compounds, DCF directly contacted the MAIT TCR (**Figure 6d**). However, the mode of DCF binding within the cleft, and subsequent contacts of DCF and MR1 with the MAIT TCR, were markedly different to the MAIT TCR-MR1-5-OP-RU interactions (**Figures 6c, d & e**). The plane of the central phenylacetic acid ring of DCF was essentially perpendicular to that of 5-OP-RU and all other MR1-ligand complexes (**Figures 6c, d & f**). Moreover, due to the flexibility in the primary amine linker of DCF, the dichlorophenyl and phenylacetic acid rings were positioned approximately orthogonal to each other (**Figure 6a**). Here, the dichlorophenyl group was buried towards the base of the cleft, wedged between Tyr7, Tyr62, Trp156 and Trp164 (**Figure 6a**). One of the chloro groups pointed towards Arg94, whereas the other was shielded by Tyr62 and Trp164 and formed a halogen bond with Lys43 (**Figure 6a**). The phenylacetic acid group is sandwiched between Arg9 and Tyr62, with the acetic acid moiety salt-bridging to Arg9 and forming a hydrogen bond with Ser24 (**Figure 6a**). Notably, to accommodate the dichlorophenyl ring of DCF within the cleft, MR1 underwent some conformational changes, including a slight reorientation of Tyr7, and, most notably, Trp69 flipping out of the way to avoid steric clashes with the ligand (**Figures 6c & d**). The reconfiguration of Trp69 had a knock-on effect in that it caused a remodeling of the CDR3β loop of the MAIT TCR, with Glu99β swinging down to salt bridge with Arg9 and Arg94, while Trp96β re-orientated to form novel contacts with Met72 and Trp69 of MR1 (**Figures 6c & d**). The hydroxyl moiety of Tyr95 from the CDR3α loop

was shown to play a pivotal role in interacting with the ribityl chain of 5-OP-RU (**Figure 6c**), but did not mediate contacts with DCF. Instead, the Tyr95α aromatic ring stacked against the phenylacetic acid ring, the latter also forming van der Waals contacts with Glu99β from the CDR3β loop (**Figure 6d**). The mode of binding of DCF was very similar to that of 5-OH DCF, with one notable difference. Namely, the 5-OH group of 5-OH-DCF formed a hydrogen bond with Glu99β from the CDR3β loop, and this additional contact to the TCR correlated with its agonist properties and with its ability to stimulate a specific MAIT TCR (**Figure 6e**).

Discussion

MR1 presents small molecules derived from vitamin B2 and B9 syntheses to MAIT cells [6]. To enable this, the architecture of the MR1 antigen-binding cleft is distinct from that of MHC-I and CD1 molecules[16]. Namely, the groove of MR1 possesses a constricted A´-pocket, rich in aromatic residues that sequesters the vitamin B-related ligands, and also shelters a lysine residue at its base that can covalently bind to an antigen via Schiff base formation. A central question in the emerging field of metabolite-mediated immunity is whether MR1 could present ligands distinct from the riboflavin precursors and folic acid derived metabolites. Here we show that MR1 can present a heterogeneous array of organic compounds with diverse chemical scaffolds, including some commonly prescribed therapeutics, which can modulate MAIT cell activity.

Given that pyrimidines are a widely used scaffold in therapeutics, we hypothesized that MR1 could present drugs and drug-like molecules to MAIT cells. Our targeted *in silico* analyses of the MR1 A´-pocket indicated this to be the case, and this was subsequently confirmed through a series of *in vitro*, *ex vivo* and *in vivo* assays. These MR1 binding compounds included a wide range of drugs and drug-like molecules, fragments and derivatives. Naturally, the ensuing functional and structural studies mostly focused on those ligands that most markedly impacted on MAIT cell function *in vitro* and paves the way for further functional and clinical investigations into how other compounds including drugs identified here can affect the function of the MAIT-MR1 axis. Our findings also have important implications for any future MAIT cell inhibitor-based therapy, as it demonstrates that administration of MR1 inhibitory ligands can impact on MAIT cell function in an *in vivo* setting, as judged by the infection model tested here. Indeed, this is the first description of any such *in vivo* modulation of MAIT cells. Clearly however, it would be of value to test the ability of drugs and drug like molecules to impact on MAIT cell function

using other mouse infection models. Our work also suggests that if endogenous ligands possess similar chemical scaffolds, they may too impact on MAIT cell function.

Although a diverse array of compounds was found to upregulate MR1 cell surface expression, the number of compounds that were able to stimulate MAIT cells was more limited, indicating greater restrictions on the structures of ligands that could both bind to MR1 and subsequently productively engage with the MAIT TCR. Of the ligands that were found to upregulate MR1 cell surface expression, all formed a Schiff base with MR1, underscoring the importance of forming a covalent adduct. This may be important for capturing MR1 within the cell and enabling its egress to the cell surface[25]. Moreover, the various non-stimulatory ligands adopted different conformations and contacts within the A´-pocket of MR1, which also correlated with their differential ability to upregulate MR1 and inhibit MAIT cell activation. For example, 3-F-SA formed additional salt-bridging interactions within the MR1 cleft, thereby accounting for its greater capacity to upregulate MR1 and its greater inhibitory potential. Notably, while 3-F-SA was non-stimulatory against the MAIT TCRs tested, 5-F-SA could weakly activate a MAIT TCR expressing cell line, indicating that subtle structural changes in the ligand, coupled with MAIT TCR heterogeneity, may lead to unanticipated MAIT cell activation. This resonates with the recent finding that diversity in the MAIT TCR repertoire can permit some folic acid derivatives to stimulate some MR1-restricted T cells and MAIT cells[22].

Of the drugs tested that activated MAIT cells, diclofenac showed the greatest potency. Its stimulatory activity was traced to naturally occurring DCF metabolites presented by MR1 on the cell surface. The mode of binding of DCF within the MR1 cleft was distinct from that of other MR1 bound ligands, not forming a Schiff base with Lys43, as also observed for some ribityllumazines[17]. Crucially, the 5-OH-DCF metabolite directly contacted the MAIT TCR α and β-chains, which provided insight into both its antigenicity and the ability of various DCF metabolites to activate specific MAIT TCRs[22], suggesting MAIT cell subset specific activation by DCF metabolites.

An important consideration, beyond the scope of this conceptual study, is whether the prescribed drugs that modulate MAIT cell activity manifest clinically in significant side effects. While the degree to which methotrexate and aminopterin degrade to 2,4-DA-6-FP in patients is unclear, high dose chemotherapeutic administration of methotrexate can result in much higher plasma concentrations of the drug than what is required to upregulate MR1 [26]. In addition, we speculate that MR1 tetramers loaded with 2,4-DA-6-FP may bind some MAIT cells, consistent with the presence of some MR1 reactive T-cells recognising 6-

FP[22]. Thus, patients administered with continual low-dose methotrexate during their treatment for rheumatoid arthritis,[27] other autoimmune diseases and cancers, are also continually exposed to low levels of 2,4-DA-6-FP. In contrast, our observations with DCF potentially reconcile reported immunological complications, such as severe hepatotoxicity. Indeed, DCF could activate a MAIT TCR at a concentration achievable in patients after an oral dose [28]. Interestingly, pharmacogenetic studies have reveled that DCF hepatotoxicity is much more prevalent in patients with polymorphisms in the enzymes responsible for DCF catabolism - including CYP2C8, which is responsible for conversion of DCF to 5-OH-DCF [29]. This is especially relevant when considering the liver, which contains both an inordinately high number of MAIT cells and high levels of DCF metabolism.

This is the first description in nature of drugs impacting on the function of MR1, a monomorphic Ag-presenting molecule. Unlike HLA-linked drug hypersensitivities where a drug can exert its effect by altering the repertoire of peptides bound by the HLA allomorph[30], the more constricted nature of the MR1 binding pocket limits binding to either the drug or a ligand associated with vitamin B2 synthesis but not both. Some of the compounds studied here, including salicylates, diclofenac, methotrexate and aminopterin, have long been known to elicit diverse pharmacological responses and hypersensitivities not completely understood in relation to targets attributed to the mechanisms of action.[27] Our findings here suggest a possible link between such drugs or their metabolites and MAIT cell function, and indicate a possible link between common drug (e.g. salicylate) intolerance [31] and modulation of MAIT cell activity. Future work could address clinical relevance of our observations and may call for the development of novel approaches to examine drug-mediated effects on MAIT cell activity in humans.

In summary, we have demonstrated that MR1 can interact with a heterogeneous array of small organic molecules, including some commonly prescribed human therapeutics. These interactions can positively or negatively modulate MAIT cell activity, thereby suggesting previously unknown immunomodulating properties of certain drugs, metabolites and small organic compounds.

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Materials and Methods

In silico virtual screening

Fragment-, shape- and receptor-based virtual screening was performed. In fragment-based screening, an in-house chemical database (~6,000 compounds) was searched for pyrimidines, enones, quinones, chromones (for example, flavones and isoflavones), as well as aromatic aldehydes, carboxylic acids and their derivatives that could potentially neutralize Lys43 through Schiff base formation or ionic contacts, respectively. This search yielded 147 virtual hits, including three FDA approved drugs (Coumarin, Thioguanine, Benzbromarone). In shape-based screening, ROCS (v 3.1.2, OpenEye Scientific Software Inc., Santa Fe, NM, http://www.eyesopen.com) [32] and Maestro Shape Screen (v 9.4, Schrödinger, NY) were used to search for analogous shapes to 6-FP from 1,216 FDA approved drugs (FDAMDD_v3b_1216_15Feb2008.sdf from http://www.epa.gov/ncct/dsstox/ DataFiles.html). 2D coordinates of 1216 drugs were converted to 3D structures with LigPrep (Schrödinger v 9.4). 16 virtual hits were obtained after visual inspection of the top 100 drugs (Combo score, ROCS; shape similarity score, Maestro), two of which were also identified in fragment-based screening (Coumarin and Thioguanine). In addition, Maestro Shape Screen was used to search for analogous shapes to 5-OP-RU from the same drug database (**Supplementary Figure 1a**). In receptor-based structure screening, GOLD (v 5.1) and Glide (v 9.4) were used to dock a pre-filtered subset of 470 of 1,216 drugs (150 < MW < 300 Da) into the 5-OP-RU binding site of the MR1-MAIT TCR crystal structure (PDB: 4NQC). Docked ligand poses were scored with ChemPLP, Chemscore and ASPscore in GOLD. Before the virtual screen, 5-OP-RU was re-docked into MR1 to reproduce its binding pose from the crystal structure and to identify scoring functions best able to predict the crystal pose (**Supplementary Figure 1c**). The structure PDB: 4NQD (MR1 K43A) was chosen for re-docking 5-OP-RU as there was no covalent bond to MR1. For the virtual screen, the active site was defined by a 10 Å radius around Arg9 and a rigid MR1 conformation was used for ligand docking. The top scoring 200 drugs were analyzed by visual inspection for ligand-protein interactions simulated from docking. To increase the hit rate, another docking programme, Glide, was also used in Standard Precision docking mode to score ligand binding using GScore. The active site

was the same for Glide and GOLD docking. In total 20 drug hits were selected for screening, one of which was also identified via fragment-based screening (Thioguanine).

Aminopterin photodegredation

Aminopterin powder (Sigma-Aldrich) was dissolved in phosphate-buffered saline (pH 8.5) at a concentration of 0.5mg/ml and exposed to light produced by a 2 x 38W fluorescent lamps at a distance of 40cm at room temperature. The degree of photodegradation was monitored using the method previously described [33], whereby the absorbance spectrum was analysed over 230-480nm using a PHERAstar plate reader (BMG labtech). The photodegradation reaction was deemed near-complete after 48h, which was subsequently confirmed by HPLC analysis against undegraded sample (not shown). The effect of degraded aminopterin on MR1 surface expression of C1R.MR1 cells was performed as described previously [34] using biotinylated 26.5 mAb and streptavidin-PE. Cells were treated for 3h prior to staining, flow cytometry analysis was carried out using a CyAn ADP (Beckman Coulter) and produced data analysed using FlowJo v10.1.

Compounds tested in cellular assays in vitro and ex vivo

6-FP and Ac-6-FP (Schircks Laboratories) were dissolved at 5 mM in water, supplemented with 17 mM NaOH. 5-OP-RU (as 1.52 mM stock solution in DMSO), was used by dilution in PBS to the required concentration immediately before testing (DMSO content was less than 0.01 % in final media). All other compounds were obtained commercially (Sigma-Aldrich, Fluka, Sapphire Bioscience) or synthesized in house and dissolved at 50 mM in DMSO. All compounds were diluted in PBS. For inhibitory compounds, vehicle controls (V) were prepared: V1 of ligands prepared at 5 mM in water supplemented with 17 mM NaOH, V2 of ligands prepared at 50 mM in DMSO.

Cell lines, PBMCs and monocyte derived dendritic cells

All cells were cultured in RPMI medium supplemented with 10 % fetal calf serum and serum complement (RF10 medium). Clonal C1R cells overexpressing MR1 (CIR.MR1)[35] or HLA-B*08:01 [36] have been described previously and were derived from C1R (ATCC number CRL-1993), a human B lymphoblastoid cell line (LCL). Clonal Jurkat.MAIT cells expressing the TRBV6-1 A-F7 MAIT TCR (Jurkat.MAIT-A-F7), the TRBV6-4 #6 MAIT TCR (Jurkat.MAIT-#6) or the TRBV20 C-F7 MAIT TCR (Jurkat.MAIT-CF7) [15] or the LC13 TCR and CD8αβ (Jurkat.CD8.LC13) [36] have been described previously and were derived from the human T cell line Jurkat RT3-T3.5 cells (Jurkat; ATCC number T1B-153). Clonal Jurkat76.MAIT-A-F7[15], bulk sorted Jurkat76.CD3.MAV21, Jurkat76.CD3.MAV36,

Jurkat76.CD3.MBV28[22] have been described previously and were derived from the human T cell line Jurkat76[37]. Bulk sorted SKW-3.M12-64 and SKW-3.M20-64[22] have been described previously and were derived from the human T cell line SKW-3 (DSMZ accession code ACC 53). For the generation of Bw58.CD3.MAIT-Vβ8.2, sequential retroviral transductions with CD3 genes and TCR genes were performed as described previously[38]. Briefly full-length CD3 genes (CD3 γ-, δ-, ζ and ε-chains) were designed, purchased and cloned into a self-cleaving 2A peptide-based (MSCV)-IRES-GFP (pMIG) vector[39]. After sequence verification, murine CD3 genes were transduced into the TCR-deficient Bw58 cells. GFP-high expressing cells (Bw58.CD3) were cloned by single cell FACS. Similarly full length MAIT TCR genes of a Vβ8.2 TCR derived from a TCR transgenic mouse using the canonical Vα19i (Vα19i $C\alpha^{-/-}$)[40] and the endogenous paring TCR β-chain (TRBV13-2*01 (Arden nomenclature Vβ8.2), TRBJ2-3*01, CDR3β CASGDAKLGVGAETLYF)) were cloned into pMIG vector, the sequence verified and then transduced into Bw58.CD3 cells to generate Bw58.CD3.MAIT-Vβ8.2. Similarly bulk sorted Jurkat.76.CD3.AM1, Jurkat.76.CD3.AM2, Jurkat.76.CD3.AM3 and bulk sorted SKW-3.M33-20 were generated based on TCR sequences described previously[22]. For the generation of high-level MR1 expressing Ag presenting cells (M12.C3.MR1), the same retroviral system was used. M12.C3 cells (an MHC-II deficient B-lymphoblastoid cell line) were transduced with murine MR1 (pMIG-MR1) and clones were generated by single cell FACS. PBMCs were isolated from whole blood of healthy donors and stored in liquid nitrogen (authorized by the Australian Red Blood Cross Service Material Supply Agreement with The University of Melbourne) as described previously[13]. Monocyte derived dendritic cells (mDCs) were generated from PBMCs by incubating plastic adherent PBMCs for 5 days in RF10 media supplemented with 300 U/ml hGM-CSF (Peprotech) and 100 U/ml hIL-4 (Peprotech). Plastic non-adherent PBMCs were refrozen in liquid nitrogen and thawed out one day prior to addition to mDCs as autologous lymphocytes in activation assays.

Activation of human and murine T cell lines

Human MR1 reactive T cell lines (10^5) were tested for activation by co-incubation with vehicle controls, PBS/media alone (background) or doses of compounds in the presence of C1R.MR1 cells (10^5) for 16 h in 200 µl RF10 medium. To test if inhibitors impact on the ability of the T cell lines to be activated, cells were incubated with PMA/Ionomycin (final concentration of 10 ng/ml and 1 µg/ml respectively) and the highest concentration of inhibitor. In addition a T cell line (Jurkat.CD8.LC13) restricted by a MHC-I molecule (HLA-B*08:01) was co-incubated with its cognate peptide FLRGRAYGL (FLR, 1 µM) and each of the compounds. Cells were subsequently stained with anti-CD3-PE-Cy7 (clone UCHT1, eBioscience), and anti-CD69-APC or anti-CD69-PE (clone FN50, BD Biosciences) mAbs as well as

anti-CD19-APC-Cy7 (clone SJ25C1, BD Biosciences) in the case of bulk sorted T cell lines (to distinguish antigen presenting cells) and 7AAD (BD Biosciences) for 30 min on ice, washed twice with FACS wash (2% fetal calf serum in PBS), fixed with FACS fix (2.1% glucose and 1% paraformaldehyde in PBS) and data acquired on a BD Canto II or LSR Fortessa flow cytometer and analysed using FlowJo. Activation of T cell lines was measured by an increase in surface CD69 expression of $CD3^+/GFP^{low}$ cells (clonal T cell lines) or $CD3^+/CD19^-$ cells (bulk sorted T cell lines) and fold of background MFI CD69 was calculated.

Jurkat76.MAIT-A-F7 or Bw58.CD3.MAIT-Vβ8.2 cells (10^5) were tested for activation by co-incubation with vehicle controls, PBS/media (background), doses of compounds and C1R.MR1 cells or M12.C3.MR1 cells (10^5) for 24 h in 200 µl RF10 medium. To test if inhibitors do not impact on the ability of the T cell lines to produce IL-2, cells were incubated with PMA/Ionomycin (final concentration of 10 ng/ml and 1 µg/ml respectively) and the highest concentration of inhibitor. IL-2 production was measured as a mean of T cell activation in ELISA (human: BD Biosciences OptEIA kit, mouse: anti-IL-2 mAb (clone JES6-1A12, BD Biosciences)) using 100 µl of supernatant, frozen/thawed to kill cells. In brief, IL-2 was assayed with biotinylated anti–IL-2 mAb and *o*-Phenylenediamine dihydrochloride (OPD; Sigma-Aldrich) substrate conversion by HRP-Streptavidin detected at 492 nm emission and IL-2 concentrations calculated based on IL-2 standards. For inhibition, inhibiting compounds were added to Ag presenting cells for 15 min before addition of 5-OP-RU and T cells. For MR1 blocking, 26.5 mAb or isotype control mAb were added at 20 µg/ml for 1 h to APCs prior to addition of T cells.
To determine half maximum inhibition by inhibitory compounds (IC_{50}), background activation levels were subtracted, data normalized, inhibitor concentrations transformed to log and non-linear regression of log (inhibitor) versus the normalized response determined using Prism software.

All cell lines tested negative for mycoplasma. Whilst SKW-3 cells are listed on the database of cross-contaminated or misidentified cell lines, where they are described as being contaminated with the KE-37 line, we have transfected specific TCR genes into these cells, which we then recloned and/or enriched by iterative cell sorting for TCR expression. Following these steps the cells were then validated for Ag recognition of cognate antigens in activation experiments.

Detection of up-regulation of MR1 on the cell surface

C1R.MR1 cells (10^5) were incubated with vehicle controls, PBS/media (background), and 100 μM of compounds for times as indicated in 200 μl RF10 medium per well. Then cells were stained with biotinylated anti-MR1 mAb 26.5 [41] or biotinylated isotype control on ice for 30 min, washed twice with FACS wash, and then stained with Streptavidin-PE (BD Biosciences) and 7AAD (BD Biosciences) on ice for 30 min, washed twice again, FACS fixed and data acquired on a BD LSR Fortessa flow cytometer and analysed using FlowJo. MR1 upregulation was measured by an increase in 26.5 staining of GFP^{high} cells (C1R.MR1) and fold of background MFI 26.5 was calculated.

Cellular screening of compounds for MR1 upregulation and Jurkat.MAIT cell activation

Sets of drugs and other small molecules were tested in a combined MAIT cell activation/MR1 upregulation assay whereby C1R.MR1 cells (10^5) and Jurkat.MAIT-A-F7 cells (10^5) were co-incubated with 100 μM and 10 μM of each compound as described above. Each time, Jurkat.MAIT-A-F7 activation by 10 nM 5-OP-RU and MR1 upregulation by 100 μM 6-FP were included as positive controls and PBS as background control. Cells were stained first with biotinylated anti-MR1 mAb 26.5, anti-CD3-PE-Cy7 (clone UCHT1, eBioscience), and anti-CD69-APC (clone FN50, BD Biosciences) on ice for 30 min, washed three times with FACS wash, and then stained with Streptavidin-PE (BD Biosciences), 7AAD (BD Biosciences) on ice for 30 min, washed twice again, FACS fixed and data acquired on a BD Canto II flow cytometer and analysed using FlowJo. To allow for comparison between experiments fold of background was normalized as % CD69 upregulation by 10 nM 5-OP-RU or % MR1 upregulation by 100 μM 6-FP equating 100 % and fold of background of 1 equating 0%. Compounds that caused levels of Jurkat.MAIT activation/MR1 upregulation of larger than 75% at 100 μM and/or 25% at 10 μM compound were considered as agonistic/stronglyMR1 upregulating and those that caused levels of activation/MR1 upregulation of larger than 7.5% and less than 75% at 100 μM and/or larger than 7.5 % and less than 25% at 10 μM compound were considered as weakly agonistic/weakly MR1 upregulating. For some of the small molecules tested at 100 μM we observed a toxic effect that resulted in cell death identified by life-dead stain. In these cases, MR1 upregulation was more pronounced at a lower concentration of inhibitor (10 μM) than at 100 μM, e.g. 5-Hydroxy-1,4-naphtoquinone. Considering MR1 upregulation at 10 μM as part of the selection criteria in such cases thus allowed to include molecules that appeared toxic at 100 μM.

Ex vivo activation of human PBMCs assayed by intracellular cytokine staining

Per sample to mDCs (10^5), PBS (background), vehicle controls and doses of inhibitors were added, followed after 15 min by PBS or titrating amounts of 5-OP-RU. Then autologous lymphocytes (0.5 x 10^6) were added per sample. To test if inhibitors impacted on the ability of cells to produce cytokines, autologous non-plastic adherent PBMCs were incubated with PMA/Ionomycin (final concentration of 100 ng/ml and 10 μg/ml respectively) and the highest concentration of inhibitor. Upon incubation for 1 h, BrefeldinA (final concentration of 10 μg/ml) was added and incubation continued for 6 h, then surface stained for 20 min at room temperature (Zombie Yellow Fixable Viability Kit (Biolegend); anti-CD3-PE-CF594 (clone UCHT1, BD Biosciences); anti-TRAV1-2-APC (clone 3C10, Biolegend); human MR1-5-OP-RU tetramer, generated as described previously[15], followed by adding PFA (final concentration of 1%) and a 20 min incubation at RT, washed twice with PBS and stained intracellularly (anti-IFNγ-Fitc (clone 25723.11, BD Biosciences); anti-TNFα-Pacific Blue (clone Mab11, Biolegend)) in 0.3% Saponin/PBS over night at 4°C. The next day, cells were washed twice in PBS and data was acquired using a BD LSR Fortessa flow cytometer and analysed by FlowJo. Percent inhibition was calculated as 100-(a/b*100), whereby 'a' represents background (% cytokine production/% CTV dilution in the presence of inhibitor only) subtracted activation by 5-OP-RU in the presence of an inhibitor and 'b' represents background (% cytokine production/% CTV dilution in the presence of PBS only) subtracted activation by 5-OP-RU.

Ex vivo activation of human PBMCs assayed by proliferation

Autologous lymphocytes and mDCs were stained with CellTrace Violet (Molecular Probes) according to the manufacturer's protocol. Per sample to mDCs (10^5), PBS (background), vehicle controls and doses of inhibitors were added, followed after 15 min by PBS or titrating amounts of 5-OP-RU. Then autologous lymphocytes (10^6 per sample) were added. To test if inhibitors impact on the ability of cells to produce cytokines, autologous non-plastic adherent PBMCs were incubated for 48 h with plate-bound anti-CD3/anti-CD28 (in house and BD Biosciences) at 10 μg/ml and at 2 μg/ml and the highest concentration of inhibitor in a flat-bottom plate before transfer to a U-shape plate after 48 h. Upon incubation of 5 days, samples were stained for 20 min at room temperature (Zombie Yellow Fixable Viability Kit (Biolegend)), followed by staining for surface markers as above in the case of ICS. Samples were washed twice with FACS wash, fixed and data was acquired using a BD LSR Fortessa flow cytometer and analysed by FlowJo. Percent inhibition was calculated as described above in the case of ICS.

Mouse in vivo activation/inhibition of MAIT cell accumulation in the lungs

Salmonella enterica serovar Typhimurium BRD509 (Salm.BRD509) has been previously described[42]. Salm.BRD509 harbours deletions in *aroA* and *aroD,* resulting in limitation of replication and spread of bacteria and has an intact riboflavin synthesis pathway. The BRD509Δ*ribDH* mutant (Salm.BRD509Δ*ribDH*), lacking a gene segment containing *ribD* and *ribH*, which encode key enzymes in the riboflavin synthesis pathway, was constructed by lamda red-recombinase mediated allelic replacement followed by transduction using phage P22 as previously described [15]. BRD509Δ*rib*DH cultures were supplemented with 20 µg/ml riboflavin. Bacteria were cultured at 37 °C statically, in Luria Bertani broth (LB) for 16-18 h to log-phase (OD_{600} 0.6-0.9) in the presence of antibiotics (30 µg/ml Streptomycin, 30 µg /ml Kanamycin) and riboflavin (20 µg/ml). For the infecting inoculum, bacteria were re-inoculated in pre-warmed medium for a further 2-4 h static culture (OD_{600} 0.4-0.6). With the estimation that 1 OD_{600}=$5x10^8$/ml, sufficient bacteria were washed and diluted in PBS for intranasal delivery to C57BL/6 mice. A sample of inoculum was plated onto Luria Agar supplemented with antibiotics and riboflavin for verification of bacterial concentration by counting CFU. Mice were bred and housed in the Biological Research Facility of the Peter Doherty Institute. Male mice aged 7-8 weeks were used in experiments and allocated to different groups at random (block randomization). The investigators were blinded to the group allocation when assessing the outcome. All procedures were approved by The University of Melbourne Animal Ethics Committee. Mice were intranasally inoculated using a total volume of 50 µl with Ac-6-FP ($5x10^4$ pmol and titrations in PBS/1% DMSO) or 3-F-SA ($5x10^5$ pmol and titrations in PBS/1% DMSO) at day -2 and day -1 or alternatively with vehicle control (PBS/1% DMSO). On day 0, mice were intranasally inoculated with BRD509Δ*ribDH* (2 x 10^7 cfu) and 5-OP-RU (3 pmol in PBS of a 14.1 mM stock in DMSO) and/or inhibitory antigens (or vehicle control) in 50 µl per nares. Then on day 1, day 2 and day 4 5-OP-RU and/or inhibitor were intranasally inoculated using a total volume of 50 µl. All inoculations were performed on anesthetized mice by isoflurane with an anaesthetizing machine. At day 7 mice were killed by administration of CO_2 and lungs (following heart perfusion with 10 ml cold RPMI) were harvested. To prepare single cell suspensions, lungs were finely chopped with a scalpel blade and treated with 3 mg/ml collagenase III (Worthington), 1 mg/ml DNase and 2% FCS in RPMI for 90 min at 37 °C with gentle shaking. Cells were then filtered (70 µM) and washed with FACS wash. Red blood cells were lysed from lung preparations with hypotonic buffer TAC (Tris-based Amino Chloride) for 5 min at RT and approximately $1.5×10^6$ cells were filtered (40 µm) and prepared for staining. To block non-specific staining, cells were incubated with mouse MR1-6-FP tetramer and anti-Fc receptor (2.4G2) for 15 min at RT. Cells were then incubated at RT for 30

minutes with antibodies from BD specific for CD19 (clone 1D3), CD45.2 (clone 104), TCRβ (clone H57-597) and 7AAD as well as mouse MR1-5-OP-RU tetramer[13] in FACS wash. Cells were fixed with 1 % PFA/PBS and 25-30 k blank calibration particles (BD) were added to enumerate absolute cell numbers, prior to analysis on a BD LSR Fortessa flow cytometer.

For intracellular cytokine staining, Golgi plug (BD Biosciences) was used during all processing steps. Cells stimulated with PMA/ionomycin (20 ng ml^{-1}, 1 μg ml^{-1}, respectively) for 4 h at 37 °C were included as positive controls alongside non-stimulated samples. Surface staining was performed as above, followed by intracellular cytokine staining for IL-17A (clone TC11-18H10, BD Biosciences) using the BD Fixation/Permeabilization Kit according to the manufacturer's instructions. Data analysis was performed with FlowJo software.

Generation of soluble proteins for crystallography

Soluble A-F7 MAIT TCR and MR1-β2m were folded from inclusion bodies and purified using methods based on those described previously [17]. MR1, β2m, TCR α-chain and TCR β-chain were overproduced separately as insoluble inclusion bodies in *E. coli* BL21(DE3) that had been transformed with pET plasmid containing gene of interest. Protein overproduction was induced in *E. coli* cells, cultured in LB media that had reached an optical density of 0.5, by the addition a 0.5 μM IPTG, and further culturing for 4 h at 37°C whilst shaking. Cells were harvested by centrifugation and lysed by resuspending in 1% (v/v) Triton-X, 4 mM $MgCl_2$, 10 mM DTT, 1 g/l lysozyme (Sigma-Aldrich) and 0.5 g/l DNase, and incubating for 2 h at room temperature. Inclusion bodies were harvested from the lysate by centrifugation, before being washed 4 times in 50 mM Tris (pH 8), 0.5% (v/v) Triton-X, 100 mM NaCl and 1 mM EDTA, omitting the Triton-X and NaCl from the final wash step. Lastly, the isolated inclusion bodies were resuspended in 20 mM Tris (pH 8) 6 M guanidinium, 0.5 mM EDTA and 1 mM DTT. The approximate concentration and purity of the isolated inclusion bodies was estimated using SDS-PAGE.

MAIT TCR was refolded by rapidly diluting 63 mg of α-chain and 42 mg of β-chain as inclusion bodies, into 500 ml of 100 mM Tris (pH 8.5), 2 mM EDTA, 0.4 M L-arginine, 5 M urea 0.5 mM oxidised glutathione, 5 mM reduced glutathione and 1 mM PMSF. Additionally, MR1-β2m bound to the compounds, HMB or 3-F-SA was refolded by rapidly diluting 56 mg of MR1 and 26 mg of β2m as inclusion bodies, into 400 ml of this same refold buffer described above but with the addition of 5 mg/l of the target compound. For 2,4-DA-6-FP, the refold contained 10 mg/ml of aminopterin that had been

photodegraded for 18h. All refold samples were stirred for 14 h at 4°C, prior to dialysing three times against 15 L of 10 mM Tris (pH 8). Dialysate was passed through Macro-Prep DEAE resin (Biorad) and protein was eluted using 10 mM Tris (pH 8) with 400 mM NaCl. Next, the eluate was concentrated down to 3 ml and purified by size exclusion chromatography using AKTA purification system and a Superdex200 15/60 column (GE Healthcare). Finally, the purified sample was passed over a HiTrap-Q HP (GE Healthcare) and eluted in 10mM Tris (pH8) using a gradient of 0 mM to 300mM NaCl over 20 CV. Purified protein was assessed for purity using SDS-PAGE and concentrations were calculated from A_{280} values measured using a NanoDrop-spectrophotometer (Thermo-fisher).

Crystallisation and structure determination

Purified MR1-β_2M-Ag was mixed with purified MAIT TCR in a 1:1 molar ratio and concentrated to a final concentration of 5 mg/ml. Samples were crystallised by hanging-drop vapour diffusion by mixing protein 1:1 with precipitant consisting of 100 mM BTP (pH 6-6.5), 8-20% (w/v) PEG3350 and 200 mM Na-acetate, as established previously [17]. Crystals formed over 1-5 days at 21°C. Diffraction data was collected at 100K on the Australian synchrotron MX1 and MX2 beamlines, from crystals that had first been washed in precipitant solution augmented with 10% (v/v) glycerol for cryo-protection. Measured reflections were indexed and integrated using either XDS [43] or imosflm, and scaled and merged using aimless [44]. Phases were calculated by molecular replacement using PHASER [45], whereby, the MR1 ternary complex, which had the CDR loops and ligand removed, was used as a search model (PDB ID: 4L4T [17]). Refinement was performed using phenix.refine [46], with the initial refinement rounds including simulated annealing, model building was performed with COOT [47], with MolProbity used in validation [48]. The Grade Web Server was used to generate ligand restraints for all drugs.

For the compounds DCF, 5-OH-DCF and 2-OH-1-NA, which did not refold sufficiently well *in vitro* (data not shown), a ligand-exchange approach was employed to obtain structures of MR1 presenting these molecules. Namely, when 6-FP (not shown), DCF, 5-OH-DCF, 2-OH-1-NA was added to the MAIT TCR-MR1-Ag protein solution at a concentration of 0.6-2 mM prior to crystallization, the resulting crystal structures determined from these crystals revealed the added compound at a high occupancy (**Supplementary Figure 6).** The buried surface area was calculated by the CCP4 implementation of Areaimol and molecular interactions were determined by the CCP4 implementation of CONTACT [44]. All molecular graphics were constructed using PyMOL.

Mass spectrometry analyses

C1R.MR1 cells (5 x 10^9) were treated with Ac-6-FP, 3-F-SA or DCF for 3 and 16 hours, after which the cells were washed extensively and the cell pellet snap frozen in liquid nitrogen. The plasma membrane from treated cells was then isolated using an AbCAM plasma membrane kit (Product number ab65400). MR1 was isolated from the plasma membrane fraction by immunoprecipitation using mAb 26.5 coupled to protein G-Agarose. MR1 and bound ligands were eluted from the resin by acidification and ligand containing fractions were further purified by solid phase extraction using a C_{18} (Waters) or SAX (Supel Co) stationary phase for DCF and 3-F-SA respectively. Components of interest were eluted from cartridges with 1 ml of methanol. Eluted components were dried on a SpeedVac dryer (Thermo Electron, Waltham, MA). The dried samples were resuspended in 50 µl of water/acetonitrile (95:5) for MS/MS analyses.

LC-MSMS for profiling of synthetic standards

Standards were subjected to chromatographic separations with a micropeptide trap (Grace Scientific) connected to an Everest C18 column (50 mm x 1 mm, 5 µm, Grace Davison) and a Bruker microQTOF mass spectrometer. The starting mobile phase consisted of 95% water (0.5% acetic acid, 10 mM ammonium acetate), and the metabolites were eluted using a linear gradient of 95% water to 85% acetonitrile over 25 min at a flow rate of 50µl/min. Mass spectra were collected in positive ionization mode by scanning over the range *m/z* 50 to 500.

Targeted detection of compounds using multiple reaction monitoring (MRM)-MS

A SCIEX QTRAP 5500 mass spectrometer was used for MRM detection of compounds. 20 µl samples were injected and loaded onto a trap column (200 µm×0.5 mm ChromXP C18-CL 3 µm 120 Å) at a flow rate of 10 µl/min in 98% buffer A for 10 minutes. For on-line fractionation of samples onto the mass spectrometer, samples were eluted from the trap column and over a cHiPLC column (75 µm×15 cm ChromXP C18-CL 3 µm 120 Å) at 300 nl/min under the following buffer B (95% acetonitrile, 0.1% formic acid in water) gradient conditions: 0–3 min 2–10% B, 3–33 min 10–40% B, 33–36 min 40–80% B, 36–38 min hold at 80% B, 38–39 min 80–2% B, followed by equilibration at 2% B until the end of the run at 48 min. The QTRAP 5500 was operated in MRM mode in unit resolution for Q1 and Q3, coupled to an information-dependent acquisition (IDA) criterion set to trigger an EPI scan (10,000 Da/sec; rolling CE; unit resolution) following any MRM transition exceeding 500 counts (ignoring the triggering MRM transition for 3 seconds thereafter).

For comparison of DCF from cell surface, transitions were simultaneously monitored in detecting DCF **Q1** to **Q3**: *m/z* 295→250, 4-OH-DCF *m/z* 312.0→266, 312→230, and 312→194 and 5-OH-DCF *m/z* 312→266, 312→230, 4′,5-dihydroxy-DCF 327.9→290, 327.9→282, 327.9→246, 325.7→210. Two transitions were simultaneously monitored in detecting Ac-6-FP **Q1** to **Q3**: *m/z* 234.1→190.1, *m/z* 234.1→147.1 and 3-F-SA detecting **Q1** to **Q3**: *m/z* 165.1→120.9 *m/z* 165.1→92.9. Data analysis was performed using Analyst v1.5.2.

Chemical synthesis

2,4-DA-6-FP, 4′,5-diOH-DCF, and 5-OH-DCF were synthesized as shown in **Supplementary Figure 11.**

Figure Legends

Figure 1. *In silico* virtual screening for putative MR1 ligands.

a) Fragment-based *in silico* virtual screen of an in-house chemical database (6,000 compounds) led to selection of 147 compounds relating to fragments of 5-OP-RU. b) Docking simulated binding poses (cyan) of selected fragments into MR1 at the binding site of 5-OP-RU. c) Examples of virtual hits from the fragment-based screen. d) Shape-based *in silico* virtual matching of 1,216 FDA approved drugs to 6-FP as a structural template leading to 16 drugs as virtual hits. e) Superimposition of the drugs on the shape of 6-FP. f) Examples of drugs as virtual hits from the shape-based screen. Colours in panel f correspond to superimposed structures in panel e. g) Receptor-based virtual screen for identification of drug hits as putative MR1-binding ligands. h) Docked poses of 20 virtual hits (cyan) from a subset of the pre-filtered FDA 1,216 drug database in the putative MR1-binding site defined by key protein residues labeled in yellow and shown as sticks. i) Examples of virtual hits from the receptor-based virtual screen.

Figure 2. **Drugs and drug-related molecules cause MR1 upregulation and MAIT cell inhibition**

a) Summary of the ability of drugs and drug-related molecules to upregulate MR1 on the surface of C1R.MR1 cells and activate Jurkat.MAIT-A-F7 cells as tested in a functional screen. Ligands of interest as part of this study are highlighted in bold font. **Supplementary Table 3** displays normalised Jurkat.MAIT-A-F7 activation/MR1 upregulation for each ligand assayed in a combined Jurkat.MAIT-A-F7 activation/C1R.MR1 upregulation assay. **b)** Chemical structures of MR1 ligands (i) 6-formylpterin (6-FP), (ii) acetyl-6-formylpterin, (iii) 2,4-diamino-6-formylpteridine (2,4-DA-6-FP), (iv) 3-formylsalicylic acid (3-F-SA), (v) 5-formylsalicylic acid (5-F-SA) and (vi) 2-hydroxy-1-napthaldehyde (2-OH-1-NA). **c)** MR1 surface upregulation by C1R.MR1 cells over time in the presence of 100 μM 6-FP, Ac-6-FP, 3-F-SA, and 2-OH-1-NA. Displayed is mean of triplicate samples and SEM (error bars) gMFI 26.5 fold of background for one representative of three experiments. **d)** Drug/small molecule dose dependent inhibition of Jurkat.MAIT-A-F7 cells activated by 5-OP-RU in the presence of C1R.MR1 cells and assayed by flow cytometric staining for CD69 as a marker of activation. Displayed is gMFI CD69 fold of background control for one representative of three experiments. **e**) Drug/small molecule dose dependent inhibition of Bw58.CD3.MAIT-Vβ8.2 cells activated by 5-OP-RU in the presence of M12.C3.MR1 cells, assayed for IL-2 production as a marker of activation. Displayed is one representative of 3 experiments. f) Capacity of 3-F-SA versus 5-F-SA to inhibit or activate Jurkat.MAIT-A-F7 and Jurkat.MAIT-#6 cells as in (d) Displayed is one representative of 3 experiments. D-f) 5-OP-RU activation with nil inhibitor/activator was assayed in triplicate displaying mean and SEM (error bars).

Figure 3. Diclofenac metabolites activate MAIT cell lines in an MR1-dependent manner.

a) Dose dependent activation of Jurkat76.MAIT-A-F7 cells by diclofenac in comparison to 5-OP-RU, RL-6-Me-7-OH, RL-6,7-DiMe, 6-FP, Ac-6-FP as well as competitive inhibition by Ac-6-FP and 26.5 blockage, assayed for IL-2 production as a marker of activation. Displayed is one representative of 2 experiments. **b)** Dose dependent activation of Jurkat.MAIT-A-F7 (TRBV6-1^{+}), Jurkat.MAIT-#6 (TRBV6-4^{+}) and Jurkat.MAIT-CF7 (TRBV20^{+}) cells by diclofenac in comparison to 5-OP-RU, RL-6-Me-7-OH, RL-6,7-DiMe, 6-FP, Ac-6-FP assayed for CD69 upregulation as a marker of activation in flow cytometry. Displayed is one representative of two experiments. **c)** Activation of Jurkat.MAIT-A-F7 (TRBV6-1^{+}), Jurkat.MAIT-#6 (TRBV6-4^{+}) and Jurkat.MAIT-C-F7 (TRBV20^{+}) cells by diclofenac (in comparison to Ac-6-FP) in combination with 5-OP-RU assayed for CD69 upregulation as a marker of activation in flow cytometry. Displayed is one experiment. **d**) Dose dependent activation of MAIT cell lines by diclofenac and diclofenac metabolites (in comparison to 5-OP-RU) as well as in combination with Ac-6-FP and 26.5 blockage assayed for CD69 upregulation as a marker of activation in flow cytometry. Displayed is one representative of two experiments. **e)** Chemical structures of (i) Diclofenac (DCF), (ii) 4-Hydroxy-diclofenac (4'-OH-DCF), (iii) 5-Hydroxy-diclofenac (5-OH-DCF), (iv) 4',5-Dihydroxy-diclofenac (4',5-diOH-DCF), (v) Diclofenac Acyl-β-D-glucuronide, (vi) Diclofenac Acyl-β-D-glucuronide allyl ester. **e)** Top: Extracted ion chromatograms (Q1-Q3 transition) of surface isolated ligands (red) and chemically synthesised ligand (black) Ac-6-FP, 3-F-SA, 5-OH-DFC and 4',5-diOH-DCF respectively (Note 4'-OH-DCF included in grey for comparison). Bottom: enhanced product ion (EPI) spectra of ligands of immunoprecipitated MR1 from the surface of C1R.MR1 cells for Ac-6-FP, 3-F-SA, 5-OH-DFC and 4',5-diOH-DCF respectively.

Figure 4. Drugs/Drug-related molecules inhibit MAIT cell activation *ex vivo* and *in vivo*

Dose dependent inhibition of cytokine production (**a**) or proliferation (**b**) of human MAIT cells (live, CD3^{+}, TRAV1-2^{+} 5-OP-RU-MR1-tetramer^{+}) *ex vivo* whereby monocyte derived DCs were co-incubated with autologous monocyte depleted PBMCs in the presence of 5-OP-RU and inhibitory ligands. Displayed are mean and SEM (error bars) of 5 donors (a and b). For 2-OH-1-NA, proliferative response could only be determined at a concentration of 25 μM, higher concentrations impaired with the ability to be activated by CD3/CD28 plate bound stimulation and with tetramer staining (data not shown). **c)** Inhibitory effect of intranasally administered Ac-6-FP and 3-F-SA (as shown in schematic) on MAIT cell accumulation in the lungs of C57BL/6 mice upon 5-OP-RU and Salm.BRD509Δ*ribDH* stimulus as

shown in scheme. Displayed are % MAIT cells of αβ T cells showing mean values +/- SEM as error bars of four mice (or three mice if excluded due to technical issues) for one experiment. The experiment was performed twice with similar results.

Figure 5. Inhibitors bound within the MR1 cleft.

A-E) MR1 residues (white) that contact directly 6-FP (**A**), 2,4-DA-6-FP (**B**), 2-OH-1-NA (**C**), HMB (**D**) and 3-F-SA (**E**), coloured green. **F**) Superposition of 6-FP (green), 2,4-DA-6-FP (purple), 2-OH-1-NA (yellow), HMB (slate) and 3-F-SA (orange) within the MR1 binding cleft. Black and red-dashed lines denote hydrogen bonds and salt bridges, respectively.

Figure 6. MAIT TCR-MR1 complexes with diclofenac and a specific metabolite.

A-B) MR1 residues that contact directly DCF (**A**) 5-OH-DCF (**B**). **C-E**) Interactions between the CDR3α (yellow) and CDR3β (orange) loops of the A-F7 MAIT TCR, with MR1 presenting 5-OP-RU (**C**), DCF (**D**) and 5-OH-DCF (**E**). **F**) Superimposition of 5-OP-RU (yellow) and DCF (green) bound within MR1. Colours and labels consistent with Figure 5 unless otherwise stated. Black, red and yellow-dashed lines denote hydrogen bonds, halogen bonds and salt bridges, respectively.

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Supplementary data

Supplementary Figure 1. In silico screening of MR1 ligands.

a) Superimposition of 9 drugs (sticks) on 5-OP-RU. These drugs are consistent with the shape matching to 6-FP. Colored chemical structures of drugs (e.g. Acyclovir, Lamivudine, Methotrexate) correspond to colored sticks, with other drugs shown in grey. **b)** Side view of all 20 virtual screening drug hits (shown as surfaces) in complementarity with the MR1 binding site. Carbon (white); oxygen (red); nitrogen (blue); chlorine (green); fluorine (cyan). **c)** RMSD comparison of re-docked 5-OP-RU by different scoring functions with the structure of 5-OP-RU (pdb: 4NQD) in crystal structure.

Supplementary Figure 2. MR1 upregulation and MAIT activation.

a) Graphical display of percentages MR1 upregulating versus agonistic compounds identified as part of the functional screen in Figure 2a. **b**) gMFI of 26.5 (mean of triplicate samples and SEM) and isotype control (single samples) staining at 7 hours for Nil, vehicle controls (V) and ligands (left panel). Repeat experiment of Figure 2c including 5-F-SA (right panel) showing single samples. **c**) First 2 rows: Drug/small molecule dose dependent inhibition of Jurkat.MAIT-#6 and Jurkat.MAIT-C-F7 activated by 5-OP-RU in the presence of C1R.MR1 cells and assayed by flow cytometric staining for CD69 as a marker of activation. Displayed is gMFI CD69 fold of background control for one representative of three experiments. 5-OP-RU activation with nil inhibitor/activator was assayed in triplicate displaying mean and SEM (error bars). Third row: gMFI of CD69 (mean of triplicate samples and SEM) for Nil (PBS), vehicle controls with maximum concentration of 5-OP-RU, and ligands co-incubated with PMA/Ionomycin are displayed for Jurkat.MAIT-A-F7, Jurkat.MAIT-#6 and Jurkat.MAIT-C-F7. **d**) IL-2 production in the presence of PBS, vehicle controls with maximum concentration of 5-OP-RU, and ligands co-incubated with PMA/Ionomycin. Displayed are mean of triplicate samples except for ligands co-incubated with PMA/Ionomycin where single samples are shown. **e**) Repeat experiment of Figure 2d/f including in addition gMFI of CD69 (mean of triplicate samples and SEM) for vehicle controls with maximum concentration of 5-OP-RU, ligands and ligands co-incubated with PMA/Ionomycin (left panel). In parallel the effect of ligands and vehicles on Jurkat.CD8.LC13 activation by C1R.HLA-B*08:01 in the presence of FLR peptide was tested (right panel).

Supplementary Figure 3. Scaffold classes and structures of bioassay-identified molecules.

Classification of twenty-two representative structures of active compounds (including 9 drugs) according to their chemical substructures: pyrimidines (black), phenols/anilines (green), enones (red), aromatic

aldehydes (orange), aromatic carboxylates (olive), quinones (dark blue), flavones (light blue), isoflavones (pink).

Supplementary Figure 4. Degradation of 2,4-DA-6-FP.

a) Chemical structures of aminopterin/folic acid (I) as they decompose to form respective formyl pterin (II) and aminobenzoylglutamic acid (III). The aldehyde on (II) then further degrades to carboxylic acid (IV). **b)** Absorbance spectra of aminopterin after exposure to a fluorescent lamp for 0h (green), 18h (orange) and 48h (red). **c&d)** MR1 surface upregulation by C1R.MR1 cells treated with 20μM or 2μM of photodegraded aminopterin from (**b**), shown as histogram of 26.5 (**c**) and as MFI 26.5-fold of PBS vehicle control (mean of triplicate samples with SEM). Representative of two separate experiments. **e)** Mass spectra and elemental analysis of compound extracted from MR1 refolded in the presence of photodegraded aminopterin compared with theoretical spectra for 2,4-DA-6-FP.

Supplementary Figure 5. Activation of MR1 restricted T cell lines by DCF and DCF metabolites

Effect of Diclofenac and its metabolites on MR1 restricted T cell lines and Jurkat.CD8.LC13 activation by C1R.HLA-B*08:01 co-incubated with FLR peptide. Displayed are fold of background MFI CD69 (a) or MFI CD69 (b) for one representative of two experiments.

Supplementary Figure 6. Inhibition of MAIT cell activation by drugs and drug-related molecules *ex vivo*.

a) % cytokine production gated on live, $CD3^+$, $TRAV1\text{-}2^+$ 5-OP-RU-MR1-$tetramer^-$ (representative of non MAIT T cells) or $TRAV1\text{-}2^+$ 5-OP-RU-MR1-$tetramer^+$ (MAIT cells) cells. Samples include titrating amounts of 5-OP-RU, vehicle controls in the presence of maximum concentration of 5-OP-RU, Nil (PBS), and maximum concentration of inhibitors in the presence or absence of PMA/Ionomycin stimulus. Displayed is data of one representative donor. **b)** % CTV dilution gated on live, $CD3^+$, $TRAV1\text{-}2^+$ 5-OP-RU-MR1-$tetramer^-$ (representative of non MAIT T-cells) or $TRAV1\text{-}2^+$ 5-OP-RU-MR1-$tetramer^+$ (MAIT cells) cells. Samples include titrating amounts of 5-OP-RU (triplicate samples, SEM) and Nil (triplicate samples, SEM), vehicles (triplicate samples, SEM) and maximum concentrations of inhibitors in the presence (triplicate samples, SEM) or absence (single samples) of plate bound CD3/CD28. Displayed is data of one representative donor.

Supplementary Figure 7. Inhibition of MAIT cell activation by small molecules *in vivo*.

(a) Inhibitory effect of intranasally administered Ac-6-FP and 3-F-SA on MAIT cell accumulation in the lungs of C57BL/6 mice upon 5-OP-RU and Salm.BRD509Δ*ribDH* stimulus. Matching data in Figure 4c, absolute numbers of MAIT cells and non-MAIT αβ T cells (mean values +/- SEM as error bars of four mice as well as CFU counts in the lungs are shown. (b) Repeat experiment at the maximum inhibitor concentration including in addition IL17 production by non-MAIT αβ T cells in response to Salm.BRD509Δ*ribDH* stimulus in the presence or absence of inhibitors.

Supplementary Figure 8. MR1 structures with drugs and drug related molecules.

A-F) Omit map of 2,4-DA-6-FP (A), 2-OH-1-NA (B), HMB (C), 3-F-SA (D) DCF (E) and 5-OH-DCF (F), after simulated-annealing refinement using Phenix-refine. F_o-F_c map (green mesh) contoured at 3σ. G) Overlay of all drug/drug related ternary structures.

Supplementary Figure 9. TCR contacts with MR1.

Contact regions of the CDR1α□(teal), CDR2α□(pink), CDR3α□(yellow), CDR1β□(cyan), CDR2β□(red), CDR3β□(orange) and framework residues (slate and deep purple for α- and β-chains, respectively) of A-F7 MAIT TCR on MR1 (white surface), which is presenting 5-OP-RU (A), 6-FP (B), 2,4-DA-6-FP (C), 2-OH-1-NA (D), HMB (E), 3-F-SA (F), DCF (G) or 5-OH-DCF (H).

Supplementary Figure 10. Docking of selected ligands into the MR1 binding site.

Predicted ligand binding poses from docking of *in silico* ligand hits into the 5-OP-RU binding site in MR1. Examples shown are a) 3-F-SA, b) 2-OH-1-NA, c) 1,2-Naphthoquinone-4-sulfonic acid (1,2-NQ-4-SA), d) HMB, e) DCF, f) Doxophylline, g) Floxuridine, h) Menadione, i) Aspirin. MR1 residues (yellow sticks), TCR residue Y95 (wheat stick), hydrogen bonds (black dashed lines).

Supplementary Figure 11. Chemical synthesis of metabolites.

Synthesis of 2,4-diamino-6-formylpteridine (A), 4′,5-dihydroxy diclofenac (B) and 5-hydroxy diclofenac (C).

Supplementary Table 1. Summary of *in silico* hits (147) from fragment based screening.

Name	CAS No.	MW
1-Amino-2-naphthol-4-sulfonic acid	116-63-2	239.25
1,2-Naphthoquinone-4-sulfonic acid sodium salt	521-24-4	260.20
1,2,4-Benzenetricarboxylic acid	528-44-9	210.14
1,3,5-Benzenetricarboxylic acid	554-95-0	210.14
1,4-Benzodioxan-6-carboxaldehyde	29668-44-8	164.16
1,4-Benzoquinone	106-51-4	108.09
1,4-Naphthoquinone	130-15-4	158.15
2-Acetamidobenzoic acid	89-52-1	179.17
2-Acetyl-1-methylpyrrole	932-16-1	123.15
2-Acetyl-5-methylfuran	1193-79-9	124.14
2-Acetylthiophene	88-15-3	126.18
2-Amino-3,5-dibromobenzaldehyde	50910-55-9	278.93
2-Amino-6-mercaptopurine (Thioguanine)[a]	154-42-7	167.19
2-Aminonicotinic acid	5345-47-1	138.12
2-Aminopyrimidine	109-12-6	95.10
2-Anilino-1,4-naphthoquinone	6628-97-3	249.27
2-Anisaldehyde	135-02-4	136.15
2-Anisic acid	579-75-9	152.15
2-Benzoylbenzoic acid	85-52-9	226.23
2-Biphenylcarboxaldehyde	1203-68-5	182.22
2-Biphenylcarboxylic acid	947-84-2	198.22
2-Carbethoxy-5,7-dihydroxy-4'-methoxyisofavone	15485-76-4	356.33
2-Carboxybenzaldehyde	119-67-5	150.13
2-Chloro-3-nitrobenzoic acid	3970-35-2	201.56
2-Chloro-4-nitrobenzoic acid	99-60-5	201.56
2-*Cis*,4-*trans*-abscisic acid	14375-45-2	264.32
2-furyl methyl ketone	1192-62-7	110.11
2-Hydroxy-1-naphthaldehyde	708-06-5	172.18
2-Hydroxy-1,4-naphthoquinone	83-72-7	174.15
2-Hydroxy-4-methoxybenzaldehyde	673-22-3	152.15
2-Hydroxy-5-methoxybenzaldehyde	672-13-9	152.15
2-Hydroxyphenylacetic acid	614-75-5	152.15
2-Methyl-1,4-naphthoquinone (Menadione)[a]	58-27-5	172.18
2-Pyrazinecarboxylic acid	98-97-5	124.10
2-Quinoxalinecarboxylic acid	879-65-2	174.16
2,3-Pyridinedicarboxylic acid	339155-13-4	167.12
2,4-Diamino-6-formylpteridine	4261-17-0	190.16
2,5-Dimethyl-p-benzoquinone	137-18-8	136.15
2,6-Dichloroquinone-4-chloroimide	101-38-2	210.45
2,6-Pyridinedicarboxylic acid	499-83-2	167.12
3-Acetylbenzonitrile	6136-68-1	145.16
3-Acetylcoumarin	3949-36-8	188.18
3-Amino-2-naphthoic acid	5959-52-4	187.19
3-Anisaldehyde	591-31-1	136.15
3-Benzyloxy-4-methoxybenzaldehyde	6346-05-0	242.27
3-Chloro-2-nitrobenzoic acid	4771-47-5	201.56
3-Chlorosalicylic acid	1829-32-9	172.57
3-Ethoxy-4-hydroxybenzaldehyde	121-32-4	166.17
3-Formylsalicylic acid	610-04-8	166.13
3-Hydroxy-4-methyl-2-nitrobenzoic acid	6946-15-2	197.14
3-Hydroxybenzoic acid	99-06-9	138.12
3-Hydroxyflavone	577-85-5	238.24
3,4-Dichlorobenzaldehyde	6287-38-3	175.01
3,4-Pyridinedicarboxylic acid	490-11-9	167.12
3,4,5-Trihydroxybenzaldehyde	13677-79-7	154.12
4-Acetamidobenzaldehyde	122-85-0	163.17
4-Acetylpyridine	1122-54-9	121.14
4-Aminouracil	873-83-6	127.10
4-Anisaldehyde	123-11-5	136.15
4-Benzyloxy-3-methoxybenzaldehyde	2426-87-1	242.27
4-Biphenylacetic acid	5728-52-9	212.24
4-Biphenylcarboxaldehyde	3218-36-8	182.22
4-Biphenylcarboxylic acid	92-92-2	198.22
4-Chloro-3-nitrobenzaldehyde	16588-34-4	185.56
4-Formylbenzoic acid	619-66-9	150.13
4-Guanidinobenzoic acid	16060-65-4	179.18
4-Hydroxy-3-methoxybenzaldehyde (isovanillin)	621-59-0	152.15
4-Hydroxy-3-methoxyphenylacetic acid	1131-94-8	182.17
4-Hydroxy-3-nitrobenzoic acid	616-82-0	183.12
4-Hydroxyphenylacetic acid	156-38-7	152.15
4-Phenoxybenzoic acid	2215-77-2	214.22
5-Aminonaphthalene-2-sulfonic acid	119-79-9	223.25
5-Aminosalicylic acid	89-57-6	153.14
5-Bromo-2'-deoxyuridine (Broxuridine)	59-14-3	307.10
5-Bromosalicylaldehyde	1761-61-1	201.02
5-Bromouracil	51-20-7	190.98

5-Chlorosalicylic acid	321-14-2	172.57
5-Formylsalicylic acid	616-76-2	166.13
5-Hydroxy-1,4-naphthoquinone	481-39-0	174.15
5,8-Dihydroxy-1,4-naphthoquinone	475-38-7	190.15
6-Aminonicotinic acid	7418-65-7	138.12
6-Chloro-4-hydroxycoumarine	19484-57-2	196.59
6-Chloropurine	87-42-3	154.56
7-Hydroxyflavone	6665-86-7	238.24
8-Aminonaphthalene-2-sulfonic acid	119-28-8	223.25
8-Hydroxyquinoline-2-carboxylic acid	1571-30-8	189.17
8-Hydroxyquinoline-5-sulfonic acid	207386-92-3	225.22
9-Anthraldehyde	642-31-9	206.24
Acridone	578-95-0	195.22
Adenine	73-24-5	135.13
Adenosine	58-61-7	267.24
Alloxan	50-71-5	142.07
Alpha-Ionone	127-41-3	192.30
Anthraflavic acid	84-60-6	240.21
Anthranilic acid	118-92-3	137.14
Anthraquinone-2-sulfonic acid sodium salt	131-08-8	310.26
Apigenin (4',5,7-trihydroxyflavone)	520-36-5	270.24
Baicalein (5,6,7-trihydroxyflavone)	491-67-8	270.24
Benzalacetone (4-phenyl-3-buten-2-one)	122-57-6	146.19
Benzbromarone	3562-84-3	424.08
Benzofuran-2-carboxylic acid	496-41-3	162.14
Biochanin A (5,7-dihydroxy-4'methoxyisoflavone)	491-80-5	284.26
Bumetanide	28395-03-1	364.42
Calconcarboxylic acid	3737-95-9	438.41
Chelidamic acid	138-60-3	183.12
Chromone-2-carboxylic acid	4940-39-0	190.15
Chrysin (5,7-Dihydroxyflavone)	480-40-0	254.24
Coumarin[a]	91-64-5	146.14
Dehydroacetic acid	520-45-6	168.15
Di-furan-2-yl-methanone	17920-86-4	162.14
Dicumarol (3,3'-methylenebis[4-hydroxycoumarin])	66-76-2	336.29
Duroquinone (tetramethyl-p-benzoquinone)	527-17-3	164.20
Galangin (3,5,7-trihydroxyflavone)	548-83-4	270.24
Gallocyanine (7-dimethylamino-4-hydroxy-3-oxo-phenoxazine-1-carboxylic acid)	1562-85-2	336.73
Genistein (4',5,7-trihydroxyisoflavone)	446-72-0	270.24
Homogentisic acid (2,5-dihydroxyphenylacetic acid)	451-13-8	168.15
Hypoxanthine (6-hydroxypurine)	68-94-0	136.11
Indole-2-carboxylic acid	1477-50-5	161.16
Indole-3-carboxaldehyde	487-89-8	145.16
Isophorone	78-59-1	138.21
Isophthalaldehyde	626-19-7	134.13
Isoquinoline-1-carboxylic acid	486-73-7	173.17
Isovanillic acid (3-hydroxy-4-methoxybenzoic acid)	645-08-9	168.15
Kaempferol (3,4',5,7-tetrahydroxyflavone)	520-18-3	286.24
Khellin	82-02-0	260.24
Kinetin (6-Furfurylaminopurine)	525-79-1	215.21
Kojic acid	501-30-4	142.11
Leucopterin (2-amino-4,6,7-trihydroxypteridine)	492-11-5	195.14
Luteolin (3',4',5,7-tetrahydroxyflavone)	491-70-3	286.24
Menadione sodium bisulfite	130-37-0	276.24
Morin hydrate (1',3,3',5,7-pentahydroxyflavone)	654055-01-3	302.24
Myricetin (3,3',4',5,5',7-hexahydroxyflavone)	529-44-2	318.24
Naringenin (4',5,7-trihydroxyflavanone)	67604-48-2	272.25
Phloroglucinol carbaldehyde (2,4,6-trihydroxybenzaldehyde)	487-70-7	154.12
Picramic acid (2-amino-4,6-dinitrophenol)	96-91-3	199.12
Quercetin (3,3',4',5,7-pentahydroxyflavone)	117-39-5	302.24
Rhodizonic acid	118-76-3	170.08
Tenofovir	147127-20-6	287.22
Tetrachloro-o-benzoquinone	2435-53-2	245.88
Tetrahydroxy-1,4-quinone	319-89-1	172.09
Theobromine	83-67-0	180.16
Theophylline (1,3-dimethylxanthine)	58-55-9	180.16
Thiamine hydrochloride (vatamin B1)	67-03-8	337.27
Toluquinone	553-97-9	122.12
Uridine	58-96-8	244.20
Vanillan (4-hydroxy-3-methoxybenzaldehyde)	121-33-5	152.15
Xanthopterin	119-44-8	179.14

[a] also identified as FDA drug hits in Supplementary Table 2.

Supplementary Table 2. Summary of screened and tested FDA approved drugs.

Name	CAS No.	MW	Use
Acyclovir	59277-89-3	225.21	antiviral
Amrinone	60719-84-8	187.20	pyridine phosphodiesterase 3 inhibitor
Aspirin	50-78-2	180.16	anti-inflammation, antipyretic
Azacytidine	320-67-2	244.21	antineoplastic
Azathiopurine	446-86-6	277.27	immunosuppressive antimetabolite
Chlorothiazide	58-94-6	295.72	a thiazide diuretic
Chloroxine	773-76-2	214.05	antibacterial
Coumarin[a]	91-64-5	146.14	perfumes and fabric conditioners,
Diclofenac	15307-86-5	296.15	NSAID
Diflunisal	22494-42-4	250.20	NSAID
Doxofylline	69975-86-6	266.25	treatment of asthma
Dyphylline	479-18-5	254.24	broncho- and vasodilator
Fenoprofen	31879-05-7	242.27	anti-inflammatory
Floxuridine	50-91-9	246.19	antineoplastic antimetabolite
Flucytosine	2022-85-7	129.09	antifungal
Flufenamic acid[a]	530-78-9	281.23	analgesic, anti-inflammatory
Ketorolac	74103-06-3	255.27	NSAID
Lamivudine	134678-17-4	229.26	a reverse transcriptase inhibitor
Mefenamic acid	61-68-7	241.29	NSAID
Menadione[a]	58-27-5	172.18	precursor of vitamin K2
Mercaptopurine	50-44-2	152.18	antimetabolite
Methaqualone	72-44-6	250.30	sedative-hypnotic drug
Methotrexate	59-05-2	454.44	antineoplastic
Methoxamine	390-28-3	211.26	alpha-adrenergic agonist
Methoxsalen	298-81-7	216.19	natural product targeting DNA
Nalidixic acid	389-08-2	232.24	antimicrobial
Phenazopyridine	94-78-0	213.24	used in urinary tract disorders
Phenylephrine	59-42-7	167.21	α-adrenergic receptor agonist
Pipemidic acid	51940-44-4	303.32	antibacterial
Pyrimethamine	58-14-0	248.71	folic acid antagonist
Quinethazone	73-49-4	289.74	diuretic
Salsalate	552-94-3	258.23	NSAID
Thioguanine	154-42-7	167.19	antineoplastic, antimetabolite
Triameterene[a]	396-01-0	253.26	a pteridine diuretic
Trimethoprim	738-70-5	290.32	antibacterial
Trichloromethiazide	133-67-5	380.66	A thiazide diuretic
Diclofenac Metabolites and other NSAIDs[b]			
4'-hydroxydiclofenac	64118-84-9	312.15	Metabolite of diclofenac
5-hydroxydiclofenac	69002-84-2	312.15	Metabolite of diclofenac
4',5-dihydroxydiclofenac	69002-86-4	328.15	Metabolite of diclofenac
Diclofenac Acyl-β-D-glucuronide	64118-81-6	472.27	Metabolite of diclofenac
Diclofenac Acyl-β-D-glucuronide allyl ester	698358-10-0	512.34	Metabolite of diclofenac
Celecoxib	169590-42-5	381.37	NSAID
Etodolac	41340-25-4	287.35	NSAID
Flunixin	38677-85-9	296.24	NSAID
Flurbiprofen	5104-49-4	244.26	NSAID
Indomethacin	53-86-1	357.79	NSAID
Meloxicam	71125-38-7	351.4	NSAID
Rofecoxib	162011-90-7	314.36	NSAID
Piroxicam	36322-90-4	331.35	NSAID
Tenoxicam	59804-37-4	337.37	NSAID

[a] Drugs not approved in US, but approved in other countries.

[b] Metabolites of Diclofenac and COX-2 inhibitors. The latter were included after identification of diclofenac as an MR1 ligand. Note: Some drugs are no longer approved in USA but are still prescribed in other countries. E.g. Coumarin is sold in India (Dipodem), Argentina (Esberiven), Brazil (Venalot), Taiwan (Venalot Depot). Flufenamic acid is sold in Japan (Fenazol 5%), Taiwan (Flufemin). Trychloromethiazide is sold in Japan (Flutria), Taiwan (Eazide). Pipemidic acid is sold in Brazil (Balurol), Italy (Diperpen). Menadione (vitamin K3) is approved for veterinary use in countries like Germany (Vita Men), Australia (Solquin), Italy (Izokappa), Chile (Katin). (Information obtained from www.drugs.com)

Supplementary Table 3. Small molecule screening

Colour coding as in Figure 2a

Compounds		Normalized Jurkat.MAIT activation		Normalized MR1 upregulation	
Name	**Use/Origin**	**100 M**	**10 M**	**100 M**	**10 M**
1,2-Naphthoquinone-4-sulfonic acid	Colourimetric determination. Synthesis of anticancer agents.	-0.48	-0.97	10.31	2.98
1,4-Benzodioxan-6-carboxaldehyde	Intermediate.	-4.20	-1.95	1.41	0.88
1,4-Naphthoquinone	Derivatives have pharmacological properties.	7.43	0.48	-4.02	35.63
2-Amino-6-mercaptopurine	Incorparates into DNA and inhibits synthesis. Treatment of leukaemia.	-4.08	3.29	-0.43	-3.26
2-Carboxybenzaldehyde	Intermediate. Metabolite of ampicillin phthalidyl ester.	-1.06	-2.69	3.06	2.12
2-Furyl methyl ketone	Intermediate. Used in the production of the antibiotic Cefuroxime.	-0.40	-2.16	-0.46	-1.39
2-Hydroxy-1-naphthaldehyde	Intermediate. Active core of sirtinol.	-0.11	4.98	125.12	107.76
2-Pyrazinecarboxylic acid	Analogue is a urate retaining drug. Metabolite of Pyrazinamide.	-3.34	-1.77	-1.54	0.32
3-Acetylcoumarin	Intermediate.	-3.23	-3.25	0.22	1.41
3-Formylsalicylic acid	Analogue of salicylic acid.	-4.07	-3.51	124.44	51.13
4-Biphenylacetic acid	Anti-inflammatory, used in the treatment of rheumatoid arthritis.	-0.15	0.76	-0.86	-0.52
4-Guanidinobenzoic acid hydrochloride	Intermediate. Aminobenzoic acid derivatives treat inflammation.	-2.25	0.78	-2.06	-7.02
5-Aminosalicylic acid	Treatment of ulcerative colitis etc.	-1.06	-2.69	3.06	2.12
5-Bromouracil	Major chemical mutagen.	-2.95	-0.27	-6.67	-3.86
5-Chlorosalicylic acid	Intermediate.	-3.15	-2.44	0.59	0.66
5-Formylsalicylic acid	Analogue of salicylic acid.	3.68	1.86	149.23	64.85
5-Hydroxy-1,4-naphthoquinone	Colouring matter isolated from walnut shells. Used in herbal remedies.	-20.19	1.45	-34.35	38.54
6-Chloro-4-hydroxy coumarine	Coumarin is from plants.	-0.06	0.87	-6.34	1.74
7-Hydroxyflavone	Intermediate. Antifungal, analgesic.	3.07	-0.48	-12.11	-0.90
Acridone	Intermediate. Derivatives show potential as antimalarial drugs.	-5.69	-2.58	-11.03	-5.47
Acyclovir	Acyclic nucleoside used in the treatment of viral infections.	-3.41	-3.58	-2.30	-0.42
Amrinone	Phosphodiesterase inhibitor. Cardiac stimulant and vasodilator.	-2.79	-0.52	-0.54	1.02
Anthranilic acid	Dyes, drugs, perfumes and pharmaceuticals.	-3.23	-2.35	0.36	0.45
Apigenin	Wool dye. Induces autophagy in leukaemia cells. Inhibits CYP2C9.	-2.10	-2.91	75.45	28.78
Azacitidine	Antineoplastic agent. Treats acute myeloid leukaemia.	-4.88	3.32	-18.75	-17.79
Azathioprine	Immunosuppressant and antieoplastic agent - treats leukaemia.	5.22	5.30	-10.17	-2.83
Baicalein	Lipoxygenase inhibitor. Anti-inflammatory agent. In the herbal supplement Sho-Saiko-To.	4.36	-1.45	41.93	5.13
Benzbromarone	Anti-gout medication. Inhibitor of CYP2C9. Causes hepatotoxicity.	18.90	0.00	-22.68	-3.46
Biochanin A	A phytoestrogen, has putative benefits in dietary cancer prophylaxis.	0.65	-0.16	-15.50	-2.62
Bumetanide	A loop diuretic. Used in the tratment of heart failure.	-4.20	-3.94	-0.77	-1.05
Celecoxib	Analgesic and anti-inflammatory - rheumatoid and osteo-arthrisis.	-2.23	-3.16	-2.74	-3.08
Chlorothiazide	Used in the treatment of oedema.	-4.76	-5.52	1.06	1.84
Chloroxine	Treatment of amoebiasis, bacterial dysentary and skin infections.	24.14	-2.33	-17.76	-0.66
Coumarin	Perfumes. Used in the treatment of asthma and lymphedema.	-5.63	-4.83	-10.76	0.54
Dehydroacetic acid	Fungicide and bactericide. Used in processed fruit and vegetables.	-4.20	-2.26	14.24	1.54
Diclofenac	Anti-inflammatory used to treat pain and other afflictions such as gout.	80.39	4.22	-12.20	6.27
Dicumarol	Anticoagulant drug related to warfarin.	-5.73	-3.86	-2.02	4.00
Diflunisal	Analgesic, anti-inflammatory and antipyretic.	2.06	-0.67	-13.73	-1.31
Doxofylline	Used in the treatment of asthma, a bronchodilator.	-4.44	-3.13	19.88	6.08
Dyphylline	Adenosine antagonist, exhibits strong activity as a bronchodilator.	-5.04	-4.85	1.14	-0.45
Etodolac	Analgesic, anti-inflammatory and antipyretic - rheumatoid disorders.	-9.30	-3.62	-6.50	-2.40
Fenoprofen	Analgesic and anti-inflammatory - rheumatoid and osteoarthrisis.	3.11	-2.27	-3.58	-2.69
Floxuridine	Antineoplastic agent, inhibits DNA and RNA synthesis.	14.56	10.77	-0.36	-1.63
Flucytosine	Antifungal agent used in the treatment of urinary tract infections.	-4.55	-4.29	0.60	0.90
Flufenamic acid	Analgesic and anti-inflammatory. Used in rheumatic disorders.	-0.15	-3.49	-5.95	1.75
Flunixin	Anti-inflammatory used by veterinarians.	-8.50	-4.50	-14.93	-4.57
Flurbiprofen	Analgesic, anti-inflammatory and antipyretic - rheumatoid disorders.	0.63	-3.66	-5.50	-2.81
Galangin	Flavonol found in galanga root. Growth inhibitor of breast tumor cells. Antiviral, antibacetrial.	15.19	4.20	-15.41	-6.60
Genistein	Phytoestrogen; tyrosine kinase inhibitor. Antioxidant and anthelmintic.	-1.68	4.32	38.44	9.32
Indomethacin	Analgesic with anti-inflammatory and antipyretic action.	3.48	5.22	-11.39	-6.85
Kaempferol	Flavone, antioxidant. Possible cancer treatment.	2.04	6.13	-22.02	-10.51
Ketorolac	Analgesic. Inhibits synthesis of prostaglandins.	-5.89	-4.21	-8.69	-1.73
Khellin	Vasodilator (asthma treatment). Induces skin pigmentation via UV light.	-4.99	-2.83	-5.04	-2.78
Kojic acid	Antibiotic.	-3.39	-1.62	-0.80	6.00
Kabetalol	Antihypertensive agent with beta-adrenoreceptor blocking properties.	-2.97	0.61	-3.12	-1.79
Lamivudine	Antiviral used in the treamtent of AIDS and hepatitis B.	-4.21	-2.65	-2.27	-3.45
Luteolin	Flavone; antioxidant, anti-inflammatory, anti-allergic and anti-cancer.	-3.66	3.18	20.31	-4.98
m-Hydroxybenzoic acid	Intermediate for plasticisers, resins, pharmaceuticals etc.	-1.43	0.04	-2.12	-1.79
Mefenamic acid	Anti-inflammartory. Relief from pain. Used in rheumatic disorders.	-3.19	-3.23	-2.55	25.78
Meloxicam	Analgesic and anti-inflammatory. Inhibits cyclooxygenase.	-2.69	-6.02	-6.57	-3.97
Menadione	Nutritional supplement. Treatment of hypoprothrombinemia.	-11.55	-4.38	14.33	47.01
Menadione sodium bisulfite	Reduces blood clotting time. Treatment for hyperprothrombinaemia.	21.16	-0.48	20.43	129.16
Mercaptopurine	Antineoplastic agent - treatment of leukaemia. Adenine analog.	17.51	14.14	-3.52	-3.37
Methaqualone	A hypnotic, used to be used for the treatment of insomnia.	-2.34	-0.43	-3.60	-2.22
Methoxamine	A pressor agent in hytotensive states.	-0.82	-0.87	-2.58	-2.42
Methoxsalen	Increases the formation of melanin following exposure to UV light.	-4.63	-3.47	2.58	1.34
Nalidixic acid	Bactericide used in the treatment of urinary-tract infections.	-3.77	-4.57	0.42	0.64
Phenazopyridine	Used in pain relief for conditions such as cystitis and urethritis.	-2.08	-0.89	-8.57	-0.67
Phenylephrine	Treatment of hypotensive states and the relief of nasal congestion.	-5.02	-4.66	-0.84	1.61
Pipemidic acid	Intermediate. A quinolone (synthetic broad-spectrum antibacterial).	-4.45	-8.50	-16.15	-14.36
Piroxicam	Analgesic, anti-inflammatory and antipyretic - rheumatoid disorders.	-0.93	-3.58	-6.05	-3.79
Pyrimethamine	Antimalarial drug and dihydrofolate reductase inhibitor.	-5.40	-5.40	-20.14	-15.35
Quinethazone	Thiazide-like diuretic used to treat hypertension.	-3.72	-1.13	-3.02	-2.12
Rofecoxib	Analgesic and anti-inflammatory. Inhibits sysnthesis of prostaglandins.	-4.97	-2.23	-4.01	-3.29
Salsalate	Anti-inflammatory, used in the treament of arthritis.	-2.42	2.10	1.47	-0.75
Tenofovir	Anti-retroviral, blocks reverse transcriptase.	-4.59	-4.34	-2.50	-4.21
Tenoxicam	Anti-inflammatory - treats rheumatoid arthristis, osteoarthristis etc.	-1.94	-3.37	-1.85	-1.12
Tetrahydroxy-1,4-quinone hydrate	Systemic keratolytic.	11.20	0.94	33.47	4.02
Theobromine	Vasodilator, diuretic and heart stimulant.	0.41	2.69	-9.49	-10.00
Triamterene	Diuretic used in the treatment of hypertension and edema.	6.14	3.49	-3.82	-3.41
Trichlormethiazide	Antihypertensive. Diuretic used to treat oedema.	-1.62	-1.13	-5.31	-2.58
Trimethoprim	Antibacterial.	0.65	-1.29	-4.92	-1.78

Supplementary Table 4. IC50 values of the inhibitors tested

T cell line	Inhibitor	Conc. 5-OP-RU	IC50 (M)	R^2 of IC50
Jurkat.MAIT-A-F7 (TRBV6-1)	6-FP	1 nM	71.40	0.97
		0.1 nM	8.86	1.00
		0.01 nM	ND	
	Ac-6-FP	1 nM	3.91	0.99
		0.1 nM	ND	
		0.01 nM	ND	
	3-F-SA	1 nM	90.34	0.98
		0.1 nM	12.25	1.00
		0.01 nM	ND	
	2-OH-1-NA	1 nM	ND	
		0.1 nM	134.00	0.90
		0.01 nM	26.85	0.87
Jurkat.MAIT-#6 (TRBV6-4)	6-FP	1 nM	122.20	0.99
		0.1 nM	12.86	1.00
		0.01 nM	0.29	0.98
	Ac-6-FP	1 nM	0.36	1.00
		0.1 nM	ND	
		0.01 nM	ND	
	3-F-SA	1 nM	150.20	1.00
		0.1 nM	18.01	1.00
		0.01 nM	8.47	0.98
	2-OH-1-NA	1 nM	ND	
		0.1 nM	106.90	0.98
		0.01 nM	17.24	0.94
Jurkat.MAIT-C-F7 (TRBV20)	6-FP	1 nM	145.80	0.98
		0.1 nM	13.04	1.00
		0.01 nM	0.33	0.94
	Ac-6-FP	1 nM	0.42	0.99
		0.1 nM	ND	
		0.01 nM	ND	
	3-F-SA	1 nM	ND	
		0.1 nM	12.62	0.98
		0.01 nM	8.87	1.00
	2-OH-1-NA	1 nM	ND	
		0.1 nM	555.10	1.00
		0.01 nM	24.01	0.90
Bw58.CD3.MAIT-V 8.2	6-FP	10 M	101.30	0.98
		5 M	166.80	0.99
		1 M	93.35	0.89
	Ac-6-FP	10 M	31.46	0.98
		5 M	17.52	1.00
		1 M	13.94	0.94
	3-F-SA	10 M	113.50	0.89
		5 M	77.66	0.99
		1 M	27.02	1.00
	2-OH-1-NA	10 M	36.30	1.00
		5 M	34.20	1.00
		1 M	31.28	1.00

ND: Not determined when non-linear regression did not converge or was ambiguous.

Supplementary Table 5. Data collection and refinement statistics

	A-F7 TCR: MR1(HMB)	A-F7 TCR: MR1(DCF)	A-F7 TCR: MR1(2,4-DA-6-FP)	A-F7 TCR: MR1(2-H-1-NA)	A-F7 TCR: MR1(3-F-SA)	A-F7 TCR: MR1(5-OH-DCF)
Data collection						
Temperature	100K	100K	100K	100K	100K	100K
Space group	*C2*	*C2*	*C2*	*C2*	*C2*	*C2*
Cell dimensions						
a, *b*, *c* (Å)	216.2, 69.7, 142.4	212.6. 69.5, 142.9	215.3, 69.7, 142.4	216.4, 69.9, 143.1	217.1, 70.5, 143.4	213.1, 69.6, 142.4
□□□□*α*, *β*, *γ*□ (°)	90, 104.3, 90	90, 103.4, 90	90, 104.0, 90	90 104.4, 90	90.0, 104.8, 90.0	90, 103.7, 90
Resolution (Å)	52.37-2.20 (2.24-2.20)	49.0-2.70 (2.78-2.70)	45.35-2.10 (2.14-2.10)	75.15-2.10 (2.14-2.10)	53.97-1.90 (1.93-1.90)	57.87-2.50 (2.56-2.50)
R_{pim}[1]	4.8 (27.3)	8.1 (50.7)	6.5 (57.8)	5.8 (36.7)	4.5 (38.1)	4.4 (28.8)
$CC_{1/2}$	99.6 (86.0)	98.0 (71.0)	99.5 (59.7)	99.3 (82.1)	99.7 (74.6)	99.6 (87.4)
I/σ_1	10.4 (2.3)	6.6 (1.3)	8.0 (1.4)	7.9 (2.1)	11.1 (1.8)	12.3 (2.3)
Completeness (%)	99.7 (97.8)	98.6 (99.2)	100.0 (100.0)	99.8 (100)	97.6 (94.2)	98.7 (97.8)
Total N$^{o.}$ observations	448364 (19719)	154771 (12941)	524584 (25862)	445269 (22402)	722223 (35045)	273724 (14991)
N$^{o.}$ unique observations	104381 (5091)	55489 (4567)	119864 (5891)	120949 (6014)	161424 (7648)	69625 (4403)
Multiplicity	4.3 (2.9)	2.8 (2.8)	4.4 (4.4)	3.7 (3.7)	4.5 (4.6)	3.9 (3.4)
Refinement statistics						
R_{factor}[2] (%)	18.4	18.8	18.0	17.3	18.1	17.3
R_{free}[3] (%)	22.8	23.8	22.6	21.9	22.5	23.1
No. atoms						
• Protein	12788	12825	12816	12799	12855	12762
• Ligand	32	70	91	60	37	42
• Water	1067	172	882	1217	1429	437
Ramachandran plot (%)						
• Most favoured	97.0	97.0	98	98	98.0	97.5
• Allowed region	2.9	2.9	2	2	1.9	2.5
B-factors (Å^2)						
• Protein	35.1	54.4	40.4	34.4	35.3	45.9
rmsd bonds (Å)	0.011	0.004	0.009	0.010	0.008	0.06
rmsd angles (°)	1.27	0.85	1.19	1.22	1.13	0.97

[1] $R_{p.i.m} = \Sigma_{hkl} [1/(N-1)]^{1/2} \Sigma_i | I_{hkl,i} - \langle I_{hkl} \rangle | / \Sigma_{hkl} \langle I_{hkl} \rangle$

[2] $R_{factor} = (\Sigma | |F_o| - |F_c| |) / (\Sigma |F_o|)$ - for all data except as indicated in footnote 3.

[3] 5% of data was used for the R_{free} calculation

Values in parentheses refer to the highest resolution bin.

Supplementary Table 6: Buried surface area calculations of MR1-Ag:MAIT TCR interaction

		Contribution (as % of total BSA)									
	Total (Å^2)	α-Chain	β-Chain	CDR1α	CDR2α	CDR3α	α-framework	CDR1β	CDR2β	CDR3β	β-framework
A-F7 TCR: MR1(DA-6-FP)	1170.2	52.3	47.7	5.8	10.5	21.9	14.0	1.2	13.1	24.8	8.6
A-F7 TCR:MR1(2-OH-1-NA)	1235.6	48.2	51.8	7.3	10.5	19.3	11.1	0.7	12.0	28.8	10.4
A-F7 TCR:MR1(HMB)	1143.0	51.2	48.8	7.6	10.9	20.8	11.9	0.9	13.7	23.6	10.7
A-F7 TCR:MR1(3-F-SA)	1210.0	48.5	51.5	7.1	10.7	20.1	10.6	0.7	11.8	27.8	11.1
A-F7 TCR:MR1(DCF)	1206.5	49.4	50.6	8.3	11.2	18.2	11.8	2.2	12.4	25.9	10.1
A-F7 TCR:MR1(5-OH-DCF)	1161.0	48.3	51.7	7.0	11.2	18.5	11.6	4.2	13.5	25.1	8.9

Determined using the CCP4 implementation of AreaiMol

Supplementary Table 7. MAIT TCR (A-F7) contacts with MR1(DCF)

CDR	TCR	MR1	Bond
CDR1α	Gly28α	Glu160	VDW
	Phe29α^{N}	Glu160^{Oε2}	H-bond
	Phe29α	Glu160, Asn155	VDW
	Asn30α	Trp156, Tyr152, Glu160	VDW
CDR2α	Val50α	Tyr152, Asn155	VDW
	Leu51α	Leu151, Asn155	VDW
CDR2α framework	Tyr48α	His148, Tyr152	VDW
	Glu55α^{Oε1}	His148^{Nε2}	H-bond
TCR framework	Arg66α^{Nη1}	Asn155^{Oδ1}	H-bond
	Arg66α	Asn155, Glu159	VDW
CDR3α	Ser93α	Tyr62, Glu160, Trp164	VDW
	Asn94α^{O}	Arg61^{Nε}	H-bond
	Asn94α^{O}	Arg61^{Nη2}	H-bond
	Asn94α^{Oη}	Tyr62^{Oη}	H-Bond
	Asn94α	Arg61, Tyr62, Trp164	VDW
	Tyr95α^{Oη}	Trp156^{Nε1}	H-bond
	Tyr95α	Arg61, Tyr62, Leu65, Tyr152, Trp156	VDW
	Asn96α	Arg61	VDW
	Gln96α	Arg61	VDW
CDR1β	Asn30β	Met72	VDW
CDR2β	Ala50β	Gln64	VDW
	Ser51β	Gly68, Arg67	VDW
	Gly53β	Arg41	VDW
	Thr54β^{Oγ}	Gln64^{Oε1}	H-bond
	Thr54β	Gln64, Arg67	VDW
	Thr55β	Gln64	VDV
CDR2β framework	Tyr48β^{Oη}	Arg61^{Nη1}	H-bond
	Tyr48β	Arg61	VDW
	Thr55β	Gln64	VDW
	Asp56β	Gln64	VDW
CDR3β	Trp96β	Leu65, Gly68, Trp69, Met72	VDW
	Thr97β	Arg61, Leu65	VDW
	Glu99β	Arg9, Trp69, Tyr152	VDW
	Glu99β ^{Oε1}	Trp69 ^{Nε1}	H-Bond
	Glu99β ^{Oε2}	Arg9^{Nη1}, Trp69 ^{Nε1}	H-Bond
	Gly100β	Glu149, Tyr152	VDW
	Ser101β^{Oβ2}	Glu149 ^{Oε1}, Glu149^{N}	H-Bond
	Ser101β	His148, Glu149	VDW

	DCF	MR1	Bond
	C	Tyr7, Ser24, Leu66	VDW
	C1	Tyr62, Leu66	VDW
	C2	Arg9, Tyr62	VDW
	C3	Try62	VDW
	C4	Tyr7	VDW
	C5	Tyr7	VDW
	C6	Tyr7, Trp164	VDW
	C7	Leu5, Tyr7, Trp156	VDW
	C8	Tyr7, Trp156	VDW
	C9	Tyr7	VDW
	C10	Tyr62	VDW
	C11	Arg9, Tyr62	VDW
	C12	Arg9, Trp69	VDW
	C13	Arg9, Tyr62	VDW
	Cl	Tyr7, Tyr62	VDW
	Cl1	Tyr7, Arg9	VDW
	N	Tyr7, Tyr62	VDW
	O	$Arg^{N\varepsilon}$, $Arg^{N\eta 2}$	H-bond
	O	Tyr7, Arg9, Ser24, Leu66	VDW
	O1	$Ser24^{O\gamma}$	H-Bond
	O1	Tyr7, Ser24, Leu66	VDW
	DCF	**TCR**	**Bond**
	C10	Tyr95α	VDW
	C11	Glu99β, Tyr95α	VDW
	C12	Glu99β	VDW

- Atomic contacts determined using the CCP4i implementation of *CONTACT* and a cutoff of 4Å.
- Van der Waals interactions defined as non-hydrogen bond contact distances of 4Å or less.
- Hydrogen bond interactions are defined as contact distances of 3.3Å or less.
- Salt bridge interactions are defined as contact distances of 4.5Å or less.

Supplementary Table 8

MAIT TCR (A-F7) contacts with MR1(5-OH-DCF)

CDR	TCR	MR1	Bond
CDR1α	Gly28α	Glu160	VDW
	Phe29α^{N}	Glu160^{Oε2}	H-bond
	Phe29α^{O}	Asn155^{Nδ2}	H-bond
	Phe29α	Glu160	VDW
	Asn30α	Tyr152, Trp156, Glu160	VDW
CDR2α	Val50α	Leu151, Tyr152, Asn155	VDW
	Leu51α	Leu151, Lys154, Asn155	VDW
CDR2α framework	Tyr48α	His148, Tyr152	VDW
	Glu55α^{Oε1}	His148^{Nε2}	H-bond
	Glu55α	His148	VDW
TCR framework	Arg66α^{Nη1}	Asn155^{Oδ1}	H-bond
	Arg66α	Asn155, Glu159	VDW
CDR3α	Ser93α^{Oγ}	Glu160^{Oε1}	H-bond
	Ser93α	Tyr62, Glu160, Trp164	VDW
	Asn94α^{Oδ1}	Tyr62^{Oη}	H-bond
	Asn94α^{O}	Arg61^{Nε}	H-bond
	Asn94α	Arg61, Tyr62, Trp164	VDW
	Tyr95α^{Oη}	Trp156^{Nε1}	H-bond
	Tyr95α^{O}	Arg61^{Nη2}	H-bond
	Tyr95α	Arg61, Tyr62, Tyr152, Trp156	VDW
	Gln96α	Arg61	VDW
CDR2β	Ala50β	Gln64	VDW
	Ser51β	Arg67, Gly68	VDW
	Gly53β	Arg41	VDW
	Thr54β^{Oγ1}	Gln64^{Oε1}, Arg67^{Nη1}	H-bond
	Thr54β	Gln64, Arg67	VDW
CDR2β framework	Tyr48β^{Oη}	Arg61^{Nη1}	H-bond
	Tyr48β	Arg61, Gln64	VDW
	Thr55β	Gln64	VDW
	Asp56β	Gln64	VDW
CDR3β	Trp96β	Leu65, Gly68, Trp69, Met72	VDW
	Thr97β	Arg61, Leu65	VDW
	Gly98β	Leu65	VDW
	Glu99β^{Oε1}	Trp69^{Nε1}	H-bond
	Glu99β^{Oε2}	Arg9^{Nη2}, Trp69^{Nε1}	H-bond
	Glu99β	Arg9, Trp69, Tyr152	VDW
	Gly100β	Glu149, Tyr152	VDW
	Ser101β^{N}	Glu149^{Oε2}	H-bond
	Ser101β	His148, Glu149	VDW
	5-OH-DCF	**MR1**	**Bond**

	CL1	Tyr7, Lys43, Tyr62	VDW
	C9	Tyr7	VDW
	C8	Trp164	VDW
	C7	Tyr7, Trp164	VDW
	C6	Tyr7, Trp156	VDW
	C5	Tyr7, Trp156	VDW
	CL	Tyr7, Ile96, Trp156	VDW
	C4	Tyr7	VDW
	N	Tyr7	VDW
	C3	Tyr62	VDW
	C10	Tyr62, Tyr95α	VDW
	C11	Arg9, Tyr62, Tyr95α, Glu99β	VDW
	C12	Arg9, Tyr62, Glu99β	VDW
	O2	$Trp69^{N\varepsilon 1}$, $Glu99\beta^{O\varepsilon 1}$, $Glu99\beta^{O\varepsilon 2}$	H-bond
	O2	Arg9, Leu65, Trp69, Glu99β	VDW
	C13	Arg9, Tyr62	VDW
	C2	Arg9, Tyr62	VDW
	C1	Tyr62, Leu66	VDW
	C	Tyr7, Ser24, Leu66	VDW
	O1	$Ser24^{O\gamma}$	H-bond
	O1	Tyr7, Ser24, Leu66	VDW
	O	$Arg9^{N\eta 2}$, $Ser24^{O\gamma}$	H-bond
	O	Tyr7, Arg9, Leu66	VDW
	5-OH-DCF	**TCR**	**Bond**
	O2	$Glu99\beta^{O\varepsilon 1}$ $Glu99\beta^{O\varepsilon 2}$	H-bonds
	O2	Glu99β	VDW
	C11	Glu99β, Tyr95α	VDW
	C12	Glu99β	VDW

- Atomic contacts determined using the CCP4i implementation of *CONTACT* and a cutoff of 4Å.
- Van der Waals interactions defined as non-hydrogen bond contact distances of 4Å or less.
- Hydrogen bond interactions are defined as contact distances of 3.3Å or less.
- Salt bridge interactions are defined as contact distances of 4.5Å or less.